Investigating the role of Annexin A1 in obesity and associated inflammation

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Chapter 5: Annexin A1 regulates adipogenesis via differential regulation of MAPK intracellular pathways

The results observed in Chapter 4 show AC2-26 significantly regulates the expression of several genes that are involved in adipocyte differentiation. Interestingly, previous *in vivo* studies of Annexin A1 gene knockout in mice report conflicting findings. Therefore, the objective of this study was to investigate the role of Annexin A1 in adipocyte differentiation using receptor agonist and antagonists to further elucidate the changes in gene expression and protein secretion in adipocytes in obesity.

5.1 Abstract

Introduction: Adipose tissue expansion is mediated via two mechanisms; hypertrophy and hyperplasia. Dysregulated expansion observed in obesity is associated with local and systematic inflammation. Adipocyte differentiation is regulated by a range of intracellular and extracellular stimuli that induce the expression of genes involved in maturation of pre-adipocyte into a mature adipocyte phenotype. The results from chapter 4, indicate Annexin A1 (ANXA1) regulates the expression of these genes in mature obesogenic adipocytes, however, the effect of ANXA1 and/or its receptors during adipocyte differentiation is unknown.

Aim: The aim of this study was to investigate the role of ANXA1 and its receptors in adipocyte differentiation.

Methodology: Simpson Golabi Behmel Syndrome (SGBS) preadipocytes were differentiated with receptor agonist (AC2-26) and antagonists (WRW4 and BOC-2) over 14 days to reach a mature adipocyte phenotype. Morphological changes (lipid accumulation, number of lipid droplets and size of lipid droplets), mRNA expressions of adipogeneic nuclear factors, lipogenic and lipolytic enzymes and adipokines, glucose uptake and activation of proteins involved in intracellular signalling pathways were investigated. The results were statistically analysed using GraphPad prism 5.0. Statistical significance at 95% was determined using One-tailed unpaired students T-test

Results: Mature cells differentiated with WRW4, AC2-26 or WRW4 and AC2-26 accumulated significantly less lipids and altered morphology of lipid droplets compared to vehicle control. The mRNA expression of adipogeneic nuclear factors (*PPARG* and *SREBP*) lipolytic enzymes (*ACC, FASN* and *GPAM*), lipolytic enzymes (*ATGL*, *HSL* and *MGL*), adipokines (*LEP, RETN, NAMPT,ADIPOQ* and *ANXA1*) and glucose uptake

transporters (*SLC2A1* and *SLC2A4*) were significantly downregulated and glucose uptake was significantly reduced in mature cells differentiated with WRW4, AC2-26 or WRW4 and AC2-26, compared to vehicle control. Furthermore, intracellular activation of p38 mitogen activated protein (p38 MAPK) were significantly decreased in mature cells differentiated with WRW4, AC2-26 or WRW4 and AC2-26, compared to vehicle control. However, mature cells differentiated with low concentrations of BOC-2 accumulated less lipids and mature cells differentiated with high concentration of BOC-2 accumulated more lipids, compared to vehicle control.

Conclusion: This effect was only apparent during later stages of adipocyte differentiation. ANXA1 may regulate the rate of adipogenesis through differential regulation of MAPK intracellular pathways. Activation of Formyl Peptide Receptor 1 (FPR1) by AC2-26/ANXA1 decreases p38 MAPK activation thus, decreasing lipid accumulation in mature adipocytes through downregulation of genes associated with adipogenesis.

5.2 Introduction

Adipose tissue is a complex, multidepot organ, producing and secreting bioactive molecules which influence a range of physiological functions including; appetite, energy expenditure, insulin sensitivity, vascular function and immunity (Osborn, Olefsky 2012). The importance of adipose tissue for health has been demonstrated through mouse models of lipoatrophy (Gavrilova, Marcus-Samuels et al. 2000, Shimomura, Hammer et al. 1999). These mice are genetically altered to express a dominant-negative protein, A-ZIP/F. It prevents the DNA binding of B-ZIP transcription factors of both CCAAT-enhancer-binding protein (C/EBP) and JUN families, inhibiting adipogenesis, therefore, resulting in very little or no white fat tissue (Moitra, Mason et al. 1998). Transplantation of adipose tissue from healthy mice, drastically reverses hyperglycaemia, improves insulin sensitivity, decreases circulating triacylglyceride, decreases hepatic gluconeogenesis and decreases ectopic fat accumulation in these mice (Gavrilova et al. 2000). However, obese individuals carrying excess amounts of adipose tissue display similar characteristics to lipoatrophic mice and humans and are at a higher risk of developing several serious chronic diseases, such as type 2 diabetes mellitus (T2DM), cardiovascular disease, hypertension, hypercholesterolemia and hypertriglyceridemia (Greenberg, Obin 2006). This suggests, an optimal amount of adipose tissue is required for normal health, too little or too much will result in ill health.

Adipose tissue expands and remodels itself by two mechanisms; hypertrophy; increase in cell size and by hyperplasia; increase in cell number to accommodate for excess nutrients (Ali, Hochfeld et al. 2013). Dysregulated expansion of the adipose tissue through these mechanisms is associated with intracellular abnormalities such as, endoplasmic reticulum and mitochondrial stress, leading to adipocyte insulin resistance, production of

adipokines, free fatty acids and inflammatory mediators; reflecting clinical manifestations of obesity (Figure 5.1) (de Ferranti, Mozaffarian 2008). In an adult, hypertrophy of adipocytes is the most common form of storing excess lipids, whereas, hyperplasia is most common in the prepubertal age (Gustafson, Gogg et al. 2009). Although, recruitment of new adipocytes in adults is less common, a recent study demonstrated a continuous turnover of ~10%/year of new adipocytes in adults (Spalding, Arner et al. 2008). Increase in fat mass in this manner, results in a metabolically healthy, insulin sensitive obese phenotype, thus hyperplastic growth results in functionally healthy smaller adipocytes. It is estimated that 20-30% of the obese population are characterised as 'metabolically healthy but obese' (Karelis, St-Pierre et al. 2004).

Adipocyte size positively correlates with whole body insulin resistance independent of body mass index, reflective of hypertrophic obesity carrying a greater risk of metabolic complications compared to hyperplastic obesity. The reduced rate of adipocyte formation in hypertrophic obesity is due to i) inadequate preadipocyte number, as observed in obese versus (vs) lean subjects or ii) attenuated differentiation capacity of preadipocytes, thus inducing hypertrophic growth resulting in enlarged swollen adipocytes predisposed to inflammation and insulin resistance (Hirsch, Fried et al. 1989, Danforth 2000, Heilbronn, Smith et al. 2004). Failure of subcutaneous preadipocytes to differentiate has been linked to central obesity (Permana, Nair et al. 2004).

However, adipocyte hypertrophy and hyperplasia are not separate phenomena, it has been suggested they co-exist and equally contribute to the metabolic dysfunctions in obesity. Hyperplasia is associated with greater severity and less reversibility of metabolic consequences (de Ferranti, Mozaffarian 2008) . Enlargement of the adipocytes alters the phenotype of these cells, resulting in major inflammatory responses in the tissue with

increased expression and secretion of pro-inflammatory in parallel to decreased expression and secretion of anti-inflammatory cytokines and adipokines (Divella, De Luca et al. 2016) . Conclusions from *in vitro* studies suggest factors such as tumour necrosis factor α (TNFα) and insulin growth factor-1 secreted by hypertrophied adipocytes stimulate hyperplasia of themselves and adjacent cells leading to altered adipocyte size (Avram, Avram et al. 2007).

The expansion of the adipose tissue is a tightly regulated process and requires communication between the cells and communication between the cells and their surrounding environment (Ali et al. 2013). Mitogens, glucocorticoids, hormones, lipids, synthetic drugs such as, thiazolidinediones and those that lead to an increase in cyclic adenosine monophosphate induce adipogenesis (Ali et al. 2013). Glucocorticoids exert complex actions on adipocytes as chronic treatment promotes adipogenesis through induction of key transcription factors such as, peroxisome proliferator activated receptor γ (PPARγ) thus inducing lipogenesis, whereas, acute treatment promotes lipolysis (John, Marino et al. 2016).

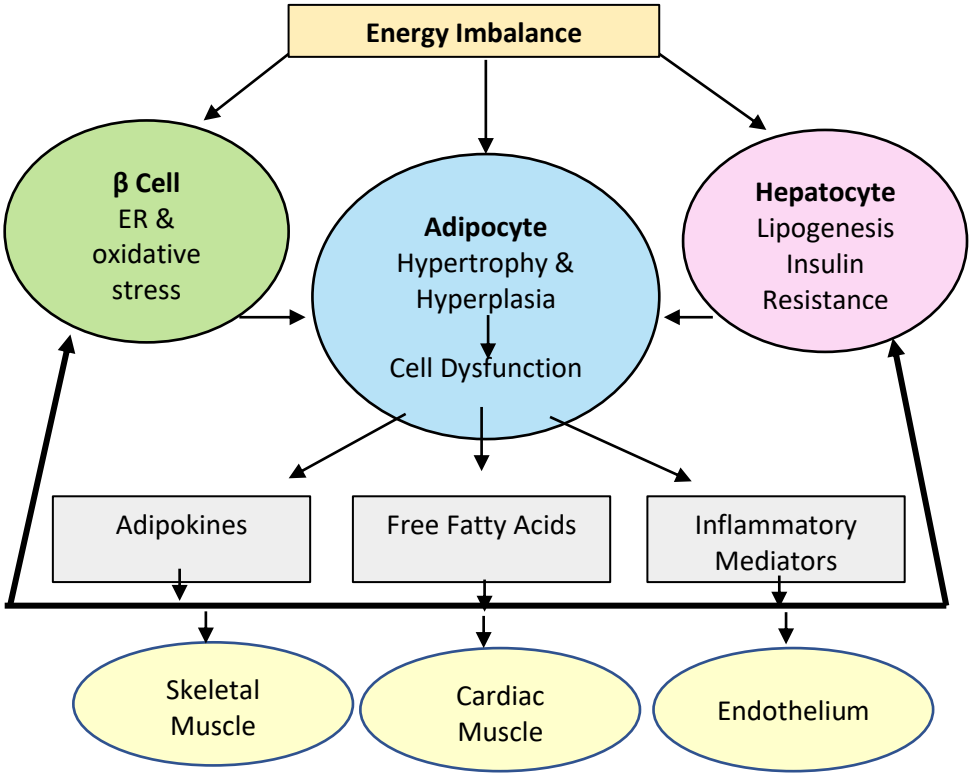


Figure 5.1: The consequences of energy imbalance. *Increased energy consumption and decreased energy expenditure affect energy homeostasis. Energy imbalance disrupts the normal function of β cells, adipocytes and hepatocytes. Chronic expansion of adipose tissue results in cell dysfunction and increased release of adipokines, free fatty acids and inflammatory mediators which affect skeletal muscle, cardiac muscle and endothelium, increasing the risk of developing Type 2 diabetes mellitus, Cardiovascular Disease and Atherosclerosis.*
ER; Endoplasmic reticulum *Adapted from (de Ferranti, Mozaffarian 2008)*

Previous *in vivo* studies of Annexin A1 (ANXA1) gene knockout in mice report conflicting findings. Decrease in number of fat cells thus, decreased weight was observed in *ANXA1-/-* mice compared to WT by (Warne, John et al. 2006), whereas, (Akasheh et al. 2013) in their study report significant increase in adiposity, blunted lipolysis and elevated hepatic lipogenesis. Furthermore, results from Chapter 4 suggest ANXA1 is involved in regulating lipid accumulation, secretion of adipokines and insulin activity and, therefore differentiation of adipocytes.

5.3 Aim, objectives and hypothesis

The aim of this study was to investigate the role of ANXA1 in the differentiation of preadipocytes into mature adipocytes.

- To assess lipid accumulation in Simpson Golabi Behmel Syndrome (SGBS) preadipocytes differentiated with and without formyl peptide receptor 2 (FPR2/ALX) antagonist and agonist
- To assess the morphology of the lipid droplets in mature SGBS adipocytes differentiated with and without FPR2/ALX antagonist and agonist
- To identify the intracellular signalling pathways activated in mature SGBS adipocytes differentiated with and without FPR2/ALX antagonist and agonist
- To assess *de-novo* lipogenesis, lipolysis and glucose uptake in mature SGBS adipocytes differentiated with and without FPR2/ALX antagonist and agonist
- To assess the gene expression of adipokines in mature SGBS adipocytes differentiated with and without FPR2/ALX antagonist and agonist

It is hypothesised that ANXA1 regulates lipid homeostasis through modulating PPARγ and is positively involved in regulating the differential process.

5.4 Materials and Methods

5.4.1 Differentiation of SGBS preadipocytes with FPR2/ALX receptor agonist and antagonists

WRWWWW (WRW4); a trp-rich hexapeptide, is a potent and specific antagonist for FRP2/ALX as it binds to and interferes with ligand-receptor interactions (IC_{50}: 230nM for agonist; WKYMVm). The antagonistic effects include, blocking ligand activated calcium increase, extracellular signal-regulated kinase (ERK) activation, chemotactic migration and internalisation in human macrophages. Similarly, a non-specific antagonist BOC-FLFLFL (BOC-2), antagonises all members of the FPR receptor family and blocks the ligand-receptor interaction inhibiting ligand induced activation of downstream signalling pathways.

5.4.1.1 Methodology

SGBS cells were differentiated using the methodology described in section 3.1.1.1.1 with the addition of 10µM WRW4 (Millipore#344220) and/or 10µM AC2-26 or dimethylsulfoxide (DMSO) (vehicle control). 10µM of WRW4 is enough to antagonise the receptor without triggering toxic effects. (Bae, Park et al. 2009). Similarly, SGBS cells were differentiated with the addition of 1µM, 10µM or 100µM BOC-2 (2BScientific#RP12950) or vehicle control. WRW4 and/or AC2-26 or BOC-2 and vehicle control were added to the cells at day 0, day 4, day 8 and day 12, coinciding with media changes. Cells were fixed and stained for analysis using the methodology described in section 3.1.2.1.1 at day 0, day 4, day 8 and day 14, or the cells were homogenised (section 3.2.1.1 (RNA) or section 5.4.5.1.1 (protein)) and the cell media were stored at -80ºC, for analysis.

5.4.2 Staining and analysis of lipid droplets in SGBS cells

Staining is a technique used in microscopy to enhance contrast in the microscopic image. Stains and dyes are used to highlight structures in biological tissues to define and examine bulk tissues, cell populations or organelles within individual cells. Lipid staining dyes include, Nile Red A; a photostable, lipophilic stain with a bright red fluorescence, Sudan III A lysochrome and Oil Red O (ORO); fat soluble dyes predominantly used for staining triglycerides in animal tissues with a maximal absorption at 518nm, stains neutral lipids and cholesteryl esters but not biological membranes (Ramirez-Zacarias, Castro-Munozledo et al. 1992).

5.4.2.1 Methodology

Prior to staining, the cells were fixed using paraformaldehyde. It causes covalent cross-links between molecules, effectively gluing them together into an insoluble meshwork. The cells were washed phosphate buffered saline (PBS) twice and incubated with fixative solution (4% paraformaldehyde and 1.5% Methanol in PBS) for 30 minutes at room temperature. The cells were washed with PBS twice and refrigerated or stained for lipid droplets.

A stock solution of ORO (Sigma#O0625) was prepared; 1mg/mL in 60% Triethyl Phosphate. The stock solution was mixed to allow the crystals to dissolve for an hour on a roller shaker at room temperature. A working solution of the dye of 60:40 in distilled water was prepared and filtered through Whatman filter paper. The cells were incubated with the dye in the dark for 2-3 hours at room temperature, washed twice with PBS to remove unbound dye and quantified for adipogenesis or stored at 4ºC.

Adipogeneic differentiation of SGBS preadipocytes for lipid quantification were carried out in 6 well plates. Approximately 8-10 images were taken per well using a light microscope at x10. The amount of ORO stain was proportional to the amount of lipids and size of adipocytes. The images were analysed using Image J following the methodology described by (Mehlem, Hagberg et al. 2013). Briefly, the images were changed from a Red-Green-Blue (RGB) into 8-bit binary images. The threshold was adjusted manually based on saturation and intensity to minimise any background staining and identify areas of interest using a control image. The threshold used in this chapter was set as, Lower limit: 33 and Upper limit: 103. The % area red was analysed per picture, as it corresponded to lipid accumulation (Chapter 5, Figure 5.2). Furthermore, the average size of lipid droplets, the average number of lipid droplets, and the average number of lipid droplets of varying sizes were analysed using the 'analyse particles' function. The sizes of the particles were manually entered as 0-10, 0-100, 100-1000 or 1000-infinity.

5.4.3 Cell viability assay

Cellular viability and toxicity can be measured by assessing various indicators including; plasma membrane integrity, DNA synthesis, DNA content, enzyme activity, presence of adenosine triphosphate (ATP) and cellular reducing conditions. Cell viability reagents such as, AlamarBlue^{TM} use the reducing power of living cells to quantitatively measure proliferation of various cells types to establish relative cytotoxicity.

The main active ingredient in AlamarBlue^{TM}; Resazurin is a non-toxic, cell permeable compound that is blue in colour and non-fluorescent. Live cells reduce compounds in their cytosol. Upon entering the cell, resazurin is reduced to resorufin, a compound red in

colour and highly fluorescent. Viable cells continue to convert resazurin, increasing the overall florescence and colour of the media surrounding the cells.

5.4.3.1 Methodology

The cellular viability was assessed using AlamarBlue™ (Invitrogen#DAL1025) by following the protocol provided by the manufacture. Briefly, SGBS preadipocytes were differentiated in 96 well plates with agonist, and/or antagonists, as described in section 5.4.1.1. On day 14, 10% AlamarBlue™ was added per well (i.e. 10µL in 100µL sample) to treated cells, positive and negative controls and incubated for 4 hours at 37ºC (95% O_2/5% CO_2). The absorbance of the reagent was measured using a spectrophotometer at 562nm, using 600nm as a reference wavelength. The standard curve generated was used to calculate % cellular viability.

5.4.4 Gene expression analysis

Cells homogenised in section were used to isolate ribonucleic acid (RNA) using methodology described in and synthesise complementary deoxyribonucleic acid (cDNA) using the methodology described in to analyse the gene expression of an array of different genes listed below:

10X *PPARG* QuantiTect Primer Assay (Qaigen#QT00029841), 10X *SREBP* QuantiTect Primer Assay (Qaigen#QT00036897), 10X *ACC* QuantiTect Primer Assay (Qaigen#QT00033761), 10X *FAS* QuantiTect Primer Assay (Qaigen#QT00014588), 10X *GPAM* QuantiTect Primer Assay (Qaigen#QT00009324), 10X *ATGL* QuantiTect Primer Assay (Qaigen#QT00019754), 10X *HSL* QuantiTect Primer Assay (Qaigen#QT00016093), 10X *MGL* QuantiTect Primer Assay (Qaigen#QT00039837), 10X *SLC2A1* QuantiTect Primer Assay (Qaigen#QT00068957), 10X *SLC2A4* QuantiTect

Primer Assay (Qaigen#QT00097902), 10X *LEP* QuantiTect Primer Assay (Qaigen#QT00030261), 10X *RET* QuantiTect Primer Assay (Qaigen#QT00210483), 10X *NAMPT* QuantiTect Primer Assay (Qaigen#QT00087920), 10X *ADIPOQ* QuantiTect Primer Assay (Qaigen#QT00014091) and 10X *ANXA1* QuantiTect Primer Assay (Qaigen#QT00078197).

5.4.4.1 Methodology

The methodology described in section was used to assess the expression of genes and the data obtained was normalised against the housekeeping gene (18s RNA) and relative gene expression was calculated using the methodology described in section 3.2.5.

5.4.5 Intracellular protein expression analysis

5.4.5.1 Protein extraction

To quantify the expression of intracellular proteins, present within the cells, the proteins need to be extracted. In general, the cell membrane is lysed by detergents, enzymes or physical agitation, sequentially precipitated and re-suspended in a suitable buffer. The concentration of the protein sample can be determined by using fluorescent assays or colorimetric assays. Extracted proteins can then be used for downstream applications such as western blots to probe against and quantify proteins of interest.

5.4.5.1.1 Methodology

Intracellular proteins were extracted using radioimmunoprecipitation assay buffer (RIPA) cell lysis buffer (Pierce#89900) following the protocol provided by the manufacturer. Briefly, SGBS cells were differentiated in 6 well plates as described in section 5.4.1.1 and on day 14, the media was decanted, and mature adipocytes were washed with ice cold

PBS twice. 100µL RIPA buffer mix (1 SigmaFast protease inhibitor cocktail tablet (Sigma#S8830) and 10 PhosSTOP tablets (Roche#04906845001) in 100mL of RIPA buffer) per well was added, and the cells were incubated for 5 minutes on ice, with gentle agitation occasionally. The cells were scrapped, and the cell suspension was centrifuged for 15 minutes at 14,000xg, 4ºC. The supernatants were transferred into new tubes and used for protein quantification (as described in section 5.4.5.2.1). Following quantification, the samples were in diluted equal volumes of Laemmli buffer (250nM Tris-hydrochloric acid-pH 6.8, 10% glycerol, 0.006% bromophenol blue, 4% sodium dodecyl sulfate, β-mercaptoethanol-pH 6.8), incubated at 100ºC for 5 minutes and stored at -20ºC.

5.4.5.2 Bicinchoninic acid assay

Protein quantification is important for standardisation of data. The basic used test for protein quantification is the biuret reaction; a chemical test used for detecting the presence of peptide bonds. In the presence of peptides, cupric form of copper is reduced to cuprous form of copper forming a violet-coloured coordination complexes in an alkaline solution. The darker the colour, the higher the protein concentration. The Pierce™ Bicinchoninic acid (BCA) is a colorimetric assay which based on the same principles. It is a two-step, high precision and a sensitive test with a high linearity rage (20-2000µg).

The first step of the assay is the binding of copper to a protein in an alkaline environment containing sodium potassium tartrate to form a light blue complex; a cuprous cation (biuret reaction). In the second step, BCA reacts with the cuprous cation formed in step one. The intense purple-colour reaction product results from binding of two BCA

molecules to one cuprous ion. The BCA/copper complex is water-soluble and exhibits a strong liner absorbance at 562nm with increasing protein concentrations.

5.4.5.2.1 Methodology

The Pierce BCA protein assay kit (Thermofisher#23227) was used to quantify protein concentrations by following the protocol provided by the manufacturer. Briefly, bovine serum albumin (BSA) was used to prepare a serial dilution (0-2000µL/mL) of known standards as described in the protocol. The BCA working reagent (WR) was prepared following a 50:1 ratio of reagent A and reagent B. 10µL of each standard and unknown sample was added in replicates into a 96 well microplate. 200µL of WR was added to each well (sample to WR ratio=1:20) and the plate was mixed thoroughly on a plate shaker for 30 seconds. The plate was covered with aluminium foil and incubated at 37ºC for 30 minutes, cooled to room temperature and measured the absorbance at 562nm on a spectrophotometer. The average absorbance measurement of blank standard was subtracted from the average absorbance measurements of all individual standards and unknown samples. A standard curve was generated, and the trend line equation used to determine the protein concentration of each unknown sample.

5.4.5.3 Western blotting

Western Blotting is an important technique used in cellular and molecular biology. It was introduced by (Towbin, Staehelin et al. 1979) and is now is used routinely for protein analysis. The specificity of the antibody-antigen interaction enables the researchers to identify specific proteins from a mixture of proteins extracted from cells.

The first step in a western blotting is separation of macromolecules in the sample using gel electrophoresis based on their molecular weight. Separated molecules are transferred

or blotted onto a second matrix; a nitrocellulose or polyvinylidene difluoride membrane. The membrane is blocked using 5% BSA or Milk powder to prevent any nonspecific binding of antibodies to the surface. The transferred protein is then probed using antibody specific to the protein of interest (primary antibody) and antibody specific to the host species of the primary antibody (secondary antibody) labelled with a conjugate molecule; biotin, fluorescent probes and enzymes. The most commonly used enzymes are horseradish peroxidase (HRP), and alkaline phosphatase (AP). The HRP-conjugated antibodies are better than AP-conjugated antibodies because HRP is smaller in size, higher activity rate and lower cost. These conjugated molecules produce a detectable signal with combined with an appropriate substrate; chromogenic, fluorescent or chemiluminescent. Use of a chemiluminescent substrate results in the production of light as a by-product (when enzyme reacts with substrate) which can be captured using film or digital imaging instruments (cameras). The data can then be quantified using densitometry software's such as, Image J.

5.4.5.3.1 Gel electrophoresis

The protein samples prepared in section 5.4.5.1.1 were further diluted with Laemmli buffer to 40µg or 60µg to a total volume of 20µL per well. The samples and precision plus protein western blotting standard (Bio-rad#1610376) were centrifuged at 1200xg for 2 minutes at 4ºC and loaded into 4-20% Precast TGX^{TM} (Tris/glycine) gradient gels (Bio-Rad, UK) in a Mini-PROTEAN^{TM} vertical electrophoresis units filled with 1x running buffer (Pierce #28362). The gels were run at 150V for 90 minutes using Power-Pac 3000 (Bio-Rad, UK).

5.4.5.3.2 Protein transfer

The protein containing polyacrylamide gels were carefully removed and sandwiched into Trans-Blot® Turbo™ transfer packs consisting of filter paper, buffer and a polyvinylidene difluoride membrane. The assembled cassettes were loaded into the Trans-Blot system (Bio-Rad, UK) and run for mixed molecular weight transfer for 7 minutes. Electrophoretic transfer is commonly used because of its speed and transfer efficiency.

5.4.5.3.3 Antibody probing

The antibodies used in this study were; anti p-ERK/total-ERK (CST#9101S/#9102S) at a concentration of 1:2000, anti-p-p38/total-p38 (NEB#9211s) at a concentration of 1:1000, anti p-ACC/total-ACC (NEB#3661s/Sigma#SAB4501395) at a concentration of 2:1000 and anti-rabbit HRP IgG (CST#7074) at a concentration of 1:10,000.

The membranes were blocked in a blocking buffer (5% w/v milk powder in tris-buffered saline (Fisher Scientific #10776834) with 1% tween 20 (TBS/T)) and incubated at room temperature for 1 hour on an orbital shaker, with the protein side facing downwards. The membranes were washed with TBS/T three times for 5 minutes each and incubated with primary antibody (5%w/v BSA in TBS/T) at 4ºC overnight on a roller shaker. The membranes were then washed with TBS/T three times for 5 minutes each to remove any unbound primary antibody and incubated with secondary antibody (5%w/v milk powder in TBS/T) at room temperature for 1 hour on an orbital shaker. The membranes were washed with TBS/T up to five times for 5 minutes each before visualising.

5.4.5.3.4 Visualisation, densitometry and quantification

The membranes were placed protein side up onto acetate sheets and 1mL of the substrate solution (1:1 solutions of SuperSignal West Femto Kit (Thermo Scientific#34095)) were

poured onto the surface of each membrane to amplify the signals. The membranes were visualised using a ChemiDoc transilluminator for imaging (Bio-Rad, UK) and visualised using the ImageLab software. The membranes were auto exposed to detect dense bands corresponding to the protein of interest, captured and analysed using Image J. The image was opened in image J and boxes were drawn around the bands of interest. The density of the band corresponds to the peak on the plot, thus the area under the peak was measured and used for data analysis.

5.4.6 Lipid peroxidation assay

Lipid peroxidation, also known as lipid oxidation, is a process of oxidative degradation of lipids in which free radicals are take electrons from lipids resulting in cell damage. Quantification of lipid peroxidation is used to assess oxidative stress. The end-product of the reaction, malondialdehyde (MDA) forms adduct with Thiobarbituric Acid (TBA) can be detected and quantified to assess the rate of lipid peroxidation.

5.4.6.1 Methodology

The lipid peroxidation assay (Abcam#136955) was carried out following the protocol provided by the manufacturer. Briefly, SGBS cells were differentiated in 96 well plates as described in section 5.4.1.1 and on day 14, mature adipocytes were washed with ice cold PBS, homogenised with 303µL lysis buffer (300µL MDA lysis buffer + 3µL butylhydroxytoluene (100X)) on ice, centrifuged at 13,000xg for 10 minutes and the supernatants were transferred into fresh Eppendorf tubes. The MDA standards (0-20 nmol) were prepared, 600µL of TBA reagent was added to each Eppendorf containing 200µL of standards and samples, incubated at 95ºC for 60 minutes and cooled to room temperature on ice for 10 minutes. 200µL of each standard and samples were plated in

triplicates in 96 well plates and absorbance were immediately measured using a spectrophotometer at optical density 532nm. The absorbance of all samples was corrected by subtracting the mean absorbance value of the blank from all standards and samples. The standard curve was generated, and the trend line equation was used to calculate the rate of lipid peroxidation in the samples.

5.4.7 Glucose uptake assay

Glucose uptake is an important biological function that can be used to study insulin activity. Glucose analog, 2-deoxyglucose (2-DG) is widely used for measuring glucose uptake because it is structurally like glucose. Like glucose, 2-DG can be taken up by glucose transporters and metabolised to 2-DG-6 phosphate (2-DG6P). However, 2-DG6P cannot be further metabolised and thus accumulates in the cells. The accumulated 2-DG6P is oxidized to generate nicotinamide adenine dinucleotide phosphate (NAPDH) resulting in an oxidized substrate, which can be measured at optical density of 412nm. The absorbance value is directly proportional to 2-DG (or glucose) uptake by the cells.

5.4.7.1 Methodology

The glucose uptake assay (Abcam#136955) was carried out following the protocol supplied by the manufacturer. Briefly, SGBS cells were differentiated in 96 well plates as described in section 5.4.1.1 and maintained for further 4 days. The cells were incubated in 100µL serum free media (Dulbecco's modified Eagle medium/Nutrient mixture F-12 + 1% anti-anti) per well overnight at 37ºC (95% O_2/5% CO_2). They were washed twice with PBS and starved for glucose by incubating in 100µL Krebs-Ringer-Phosphate-Hepes F(20mM HEPES, 5mM potassium phosphate monobasic, 1mM magnesium sulfate, 1mM calcium chloride, 136mM sodium chloride and 4.7 mM potassium chloride; pH 7.4) +

2% BSA buffer per well for 40 minutes, at 37ºC (95% O_2/5% CO_2). The cells were stimulated with 1µM insulin, 10µM WRW4, 10µM AC2-26 or vehicle controls and incubated at 37ºC (95% O_2/5% CO_2) for 20 minutes, followed by 10mM 2-DG incubation at 37ºC (95% O_2/5% CO_2) for a further 20 minutes. A 2-DG6P standard curve (0-100pmol/well) was prepared and NADPH was generated from 2DG6P. Endogenous NAD(P) was degraded to reduce background signals and a recycling amplification reaction was carried out and the uptake of 2-DG was measured at 412nm using a spectrophotometer in a kinetic mode, every 5 minutes for 20 minutes. The absorbance of all samples was corrected by subtracting the mean absorbance value of the blank from all standards and samples. The standard curve was generated, and the trend line equation was used to calculate the amount of 2-DG6P in the samples.

5.4.8 Data and statistical analysis

The data in this study is presented as Mean ± Standard Error of the Mean (SEM). One tailed, unpaired student's T-test was used to statistically analyse the data and to assess the differences in the data sets, a p value of $p<0.05$ was considered statistically significant. GraphPad Prism 5 was used to statistically analyse the data and produce the graphs.

5.5 Results

5.5.1 Lipid accumulation in SGBS cells differentiated with and without FPR2/ALX receptor antagonists and/or agonist

SGBS cells were seeded and induced to differentiate for 14 days to reach a mature adipocyte phenotype. A key phenotypic hallmark of adipocyte differentiation is the accumulation of lipid droplets. Cells at Day 0, 4, 8 and 14 were stained with ORO and assessed for lipid accumulation. Figure 5.2a displays a pictorial representation of the accumulation of lipid droplets over the 14-day period. As expected, small lipid droplets were first observed at day 4 indicative of adipogeneic capacity of these cells. At day 0, 20.5% ±2.8, n=3 of the area analysed showed lipid staining which significantly increased at day 4; 57.9% ±4.7, $p<0.0001$, n=3. Lipids continue to significantly accumulate through to day 8; 69.6% ±4.1, $p<0.001$, n=3 and significantly increased at day 14; 100% ±5.1, $p<0.0001$, n=6, as shown in Figure 5.2b.

SGBS cells were seeded and induced to differentiate for 14 days with 10 µM WRW4 or DMSO as vehicle control. Cells at Day 0, 4, 8 and 14 were stained with ORO and assessed for lipid accumulation. A significant increase in lipid accumulation at day 4 was observed in comparison to day 0; 50.2% ±4.0 vs 20.5% ±2.8 respectively, $p<0.0001$, n=3, as shown in Figure 5.3b. Similarly, a significant increase was observed at day 8; 70.8% ±3.4, $p=0.0001$, n=3, however, no significant difference was observed between day 8 and day 14. Both the vehicle control and WRW4 differentiated cells showed similar lipid accumulation pattern and phenotype at day 14. Interestingly, no significant difference in lipid accumulation between vehicle control and WRW4 differentiated cells was observed during the early stages of differentiation (Days 0-8), however, lipid accumulation

significantly decreased in WRW4 differentiated cells compared to vehicle control; 78.0% ±4.7 vs 100% ±5.1, p=0.0016, n=6, respectively at day 14, as shown in Figure 5.3b.

Inhibiting the activity of FPR2/ALX using a specific receptor antagonist demonstrated a significant decrease in lipid accumulation therefore, it was thought FPR2/ALX activation using the receptor agonist may increase lipid accumulation, suggesting it plays a positive role in adipocyte differentiation. SGBS cells were seeded and induced to differentiate for 14 days with 10µM AC2-26 or vehicle control. A significant increase in lipid accumulation at day 4 was observed in comparison to day 0; 48.9% ±4.2 vs 20.5% ±2.8 respectively, p<0.0001, n=3, as shown in Figure 5.4b. Similarly, a significant increase was observed at day 8; 70.3% ±3.6, p=0.0001, n=3, however, no significant difference was observed between day 8 and day 14. Like WRW4 differentiated cells, no significant difference in lipid accumulation between vehicle control or AC2-26 differentiated cells were observed during the early stages of differentiation (Days 0-8). However, lipid accumulation significantly decreased in AC2-26 differentiated cells compared to vehicle control; 79.6% ±3.6 vs 100% ±5.1, p=0.0013, n=6, respectively at day 14, as shown in Figure 5.4b.

Although ANXA1 is a specific agonist for FPR2/ALX, its *N* terminal peptide AC2-26 can also bind to and activate other members of the FPR family such as FPR1 (Woloszynek, Hu et al. 2012). Antagonising FPR2/ALX may redirect the binding of endogenous AC2-26 to FPR1 and instigating a decrease in lipid accumulation, as shown in Figure 5.28. Thereby, FPR1 activation with the treatment of AC2-26 may override the effects of FPR2/ALX activation presenting equivalent results. Thus, activating FPR1 and inhibiting FPR2/ALX simultaneously, may support to identify if FPR1 influences lipid accumulation, shown in Figure 5.28. Thus, SBGS cells were seeded and induced to

differentiate with 10µM WRW4 and 10µM AC2-26 or vehicle control over 14 days. The cells were stained with ORO and assessed for lipid accumulation. A significant increase in lipid accumulation at day 4 was observed in comparison to day 0; 54.5% ±4.6 vs 20.5% ±2.8 respectively, $p<0.0001$, $n=3$, as shown in Figure 5.5b. Similarly, a significant increase was observed at day 8; 73.2% ±3.7, $p=0.0030$, $n=3$, however, no significant difference was observed between day 8 and day 14. No significant difference in lipid accumulation between vehicle control and WRW4 + AC2-26 differentiated cells was observed during the early stages of differentiation (Days 0-8). However, WRW4 + AC2-26 differentiated cells accumulated significantly less lipids than vehicle control; 77.2% ±5.2 vs 100% ±5.1, $p=0.0011$, $n=6$, respectively at day 14, as shown in 5.5b.

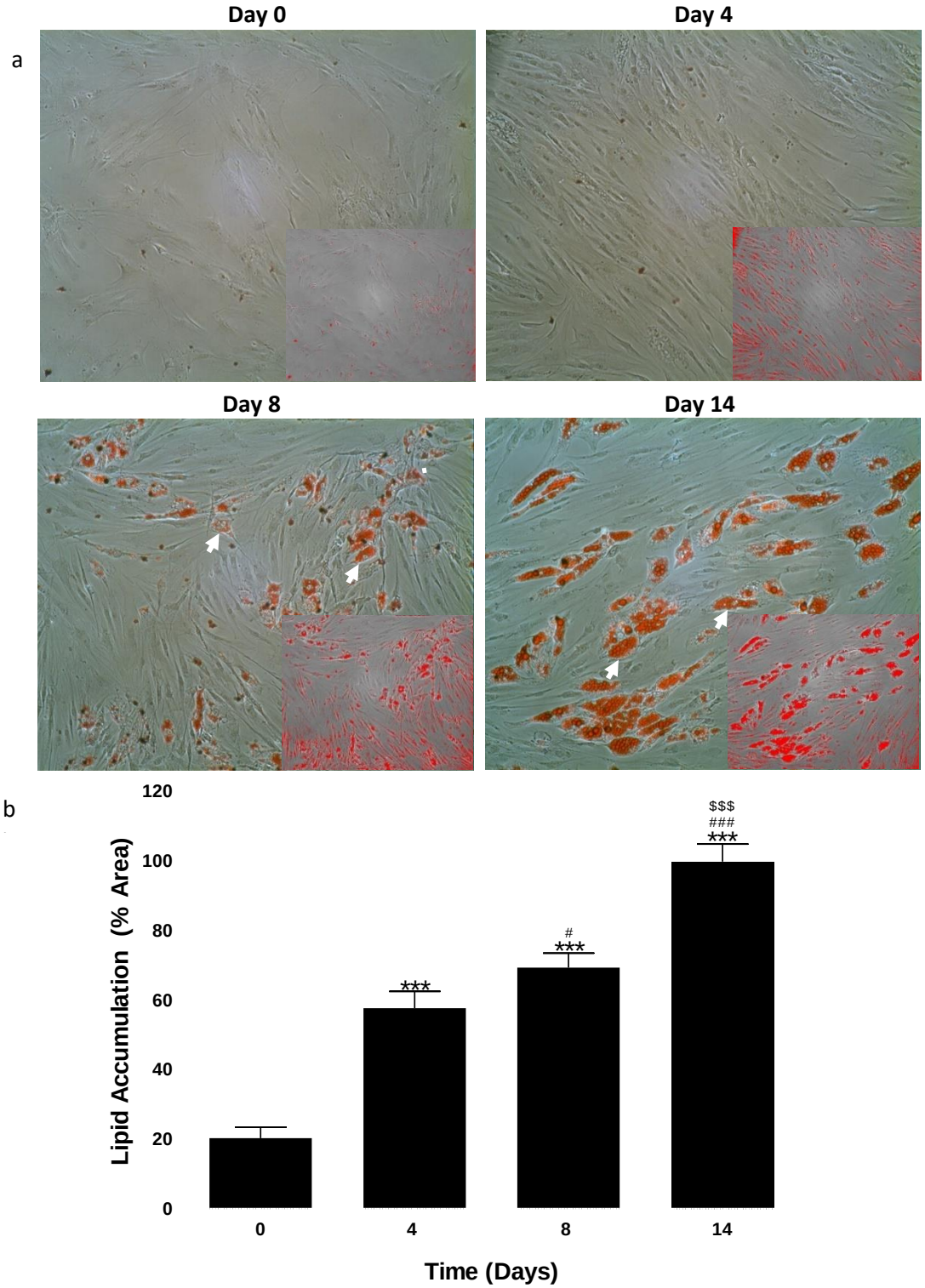
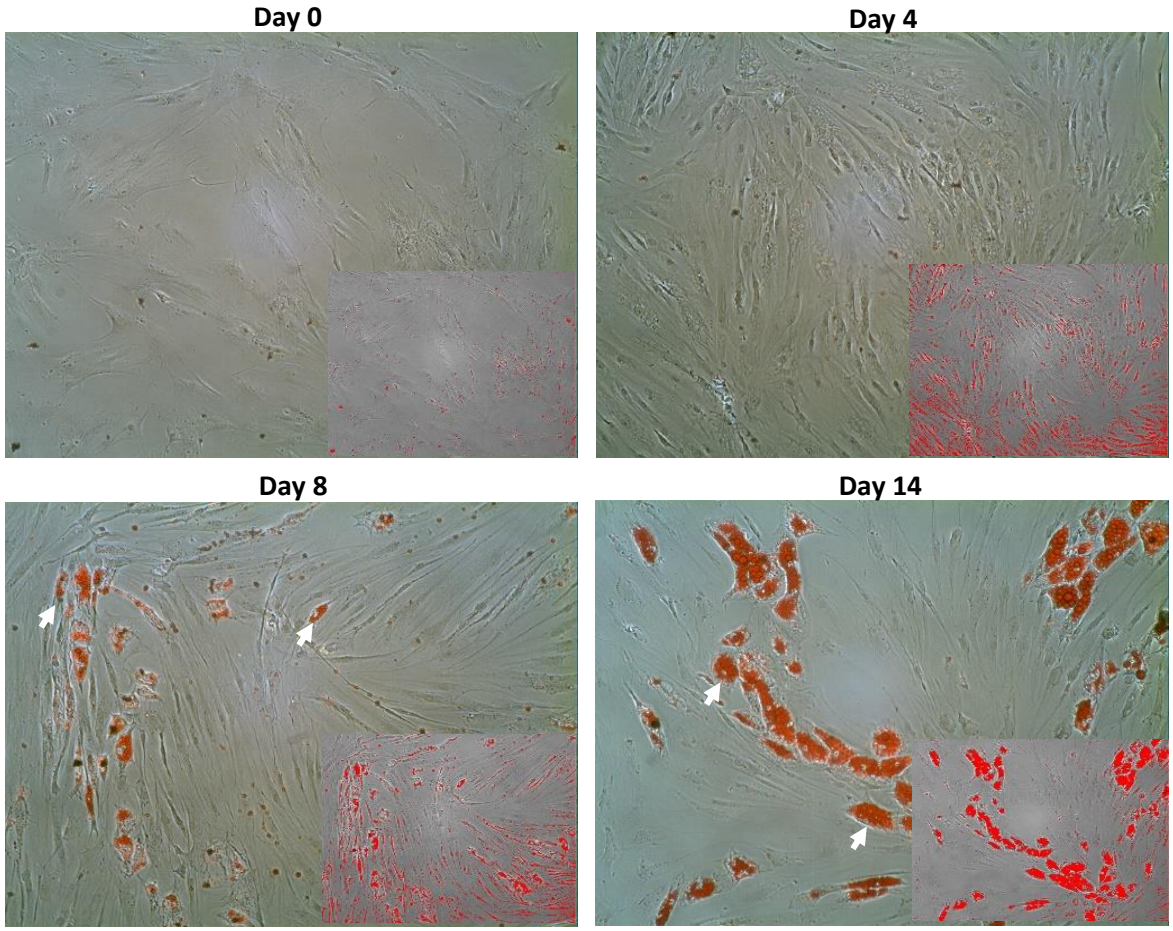


Figure 5.2: Lipid accumulation during differentiation of SGBS cells. *SGBS cells were differentiated and lipid droplets were stained using Oil Red O dye at Day 0, 4, 8 and 14. a) Visual representation of lipid droplets indicated by white arrows (magnification x10). b) Oil Red O staining was analysed using Image J. One tailed unpaired student's T-test was used to statistically analyse the data. Significant increase in lipid accumulation was observed at day 14 in comparison to day 0 ($p<0.0001$, $n=6$). Data represents as Mean ± SEM normalised to day 14. P value was set at $P<0.05$ and is donated by *= Day 0 vs, #= Day 4 vs and $= Day 8 vs.*

a)



b

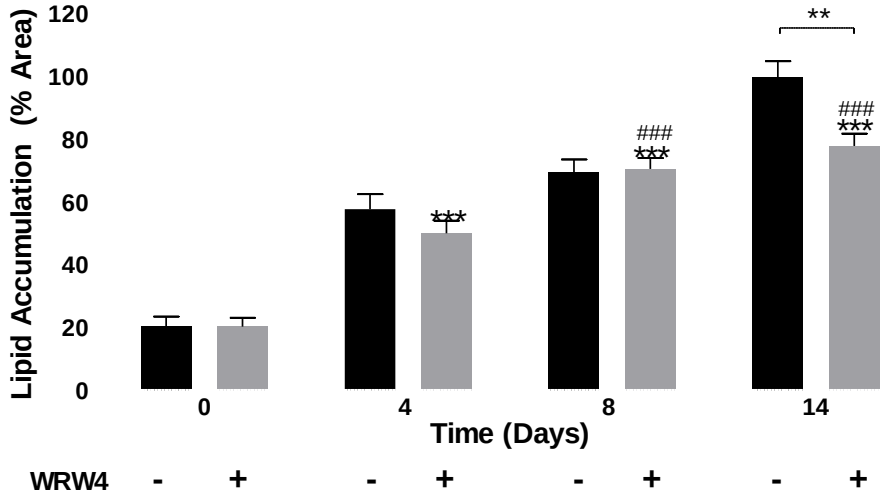


Figure 5.3: Lipid accumulation during differentiation of SGBS cells treated with FPR2/ALX antagonist; WRW4. *SGBS cells were differentiated with and without 10μM WRW4 and lipid droplets were stained using Oil Red O at Day 0, 4, 8 and 14. a) Visual representation of lipid droplets indicated by white arrows (magnification x10). b) Oil Red O staining was analysed using Image J. One tailed unpaired student's T-test was used to statistically analyse the data. Significant increase in lipid accumulation was observed at day 14 in comparison to day 0 ($p<0.0001$, $n=6$) in WRW4 differentiated cells. No significant difference in lipid accumulation was observed between vehicle control and WRW4 differentiated cells between day 0 and 8. However, at Day 14, lipid accumulation was significantly less in WRW4 differentiated cells compared to vehicle control ($p=0.0367$, $n=6$). Data represents as Mean ± SEM normalised to vehicle control. P value was set at $P<0.05$ and is denoted by ***= Day 0 vs, ####= 4 vs. Histogram: Black= control, Grey= treated*

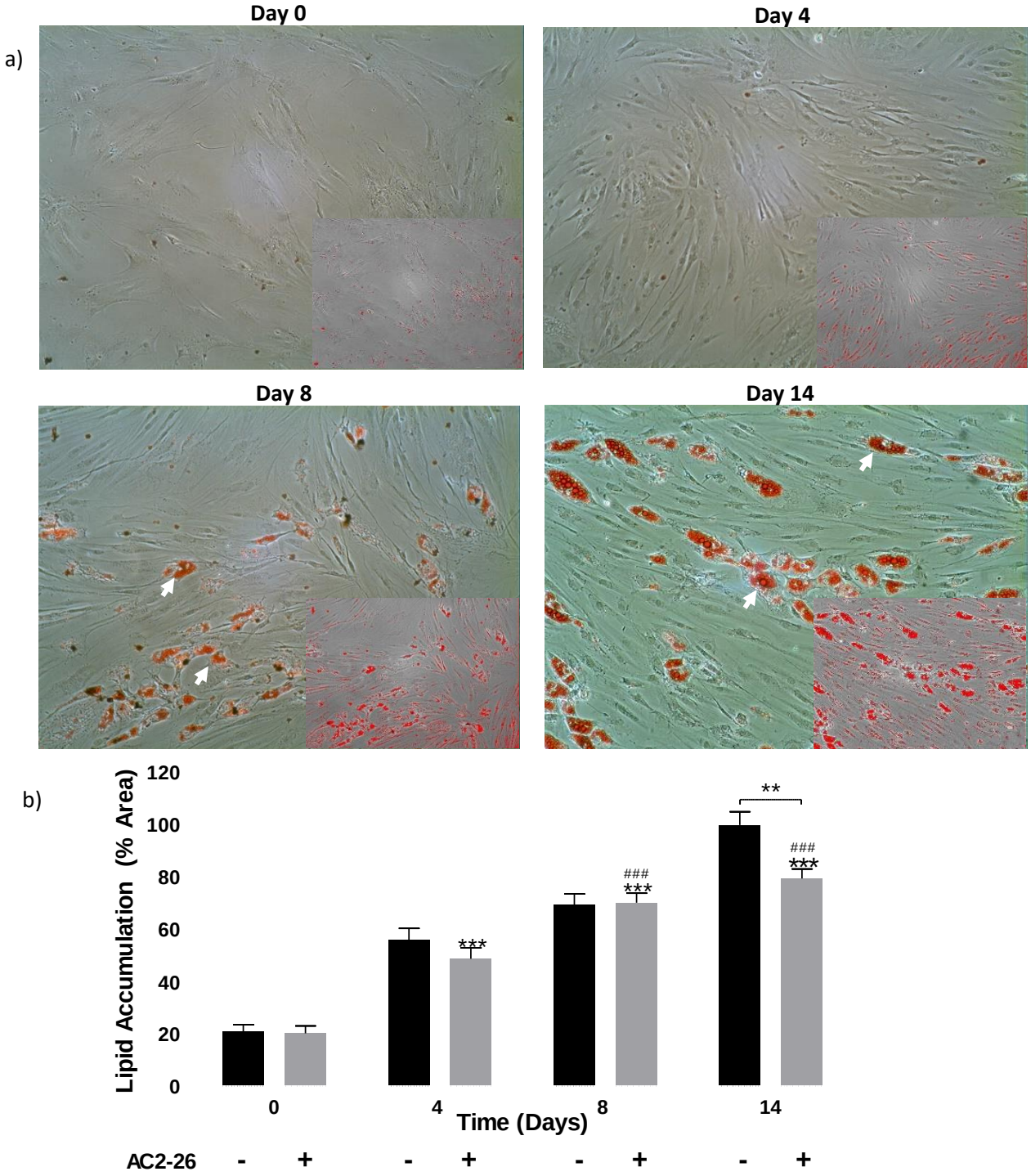
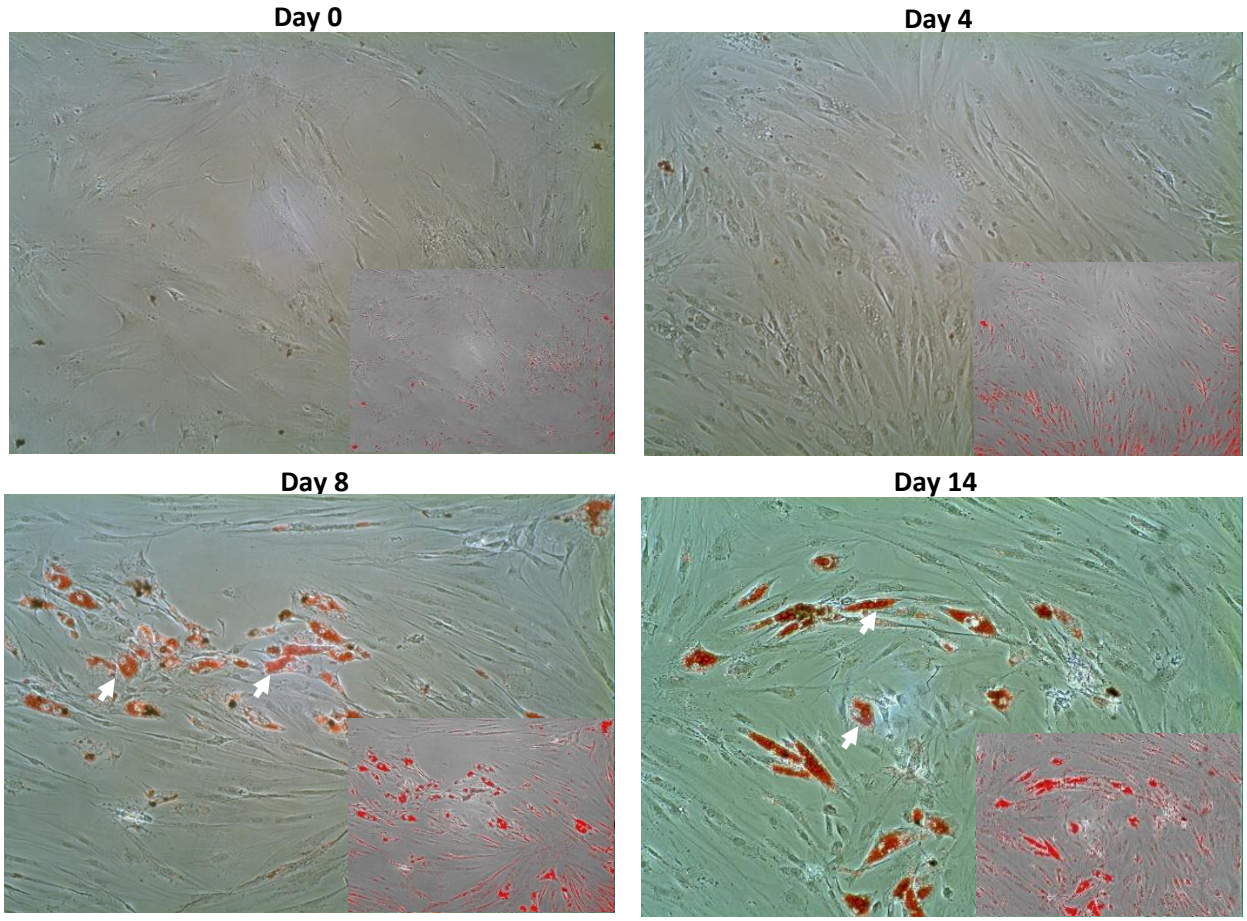


Figure 5.4: Lipid accumulation during differentiation of SGBS cells treated with FPR2/ALX agonist; AC2-26. *SGBS cells were differentiated with and without 10μM AC2-26 and lipid droplets were stained using Oil Red O at Day 0, 4, 8 and 14. a) Visual representation of lipid droplets indicated by white arrows (magnification x10). b) Oil Red O staining was analysed using Image J. One tailed unpaired student's T-test was used to statistically analyse the data. Significant increase in lipid accumulation was observed at day 14 in comparison to day 0 ($p<0.0001$, $n=6$) in AC2-26 differentiated cells. No significant difference in lipid accumulation was observed between vehicle control and AC2-26 differentiated cells between day 0 and 8. However, at Day 14, lipid accumulation was significantly less in AC2-26 differentiated cells compared to vehicle control ($p=0.0210$, $n=6$). Data represents as Mean ± SEM normalised to vehicle control. P value was set at $P<0.05$ and is denoted by ***= Day 0 vs, ####= 4 vs. Histogram: Black= control, Grey= treated*

a)



b)

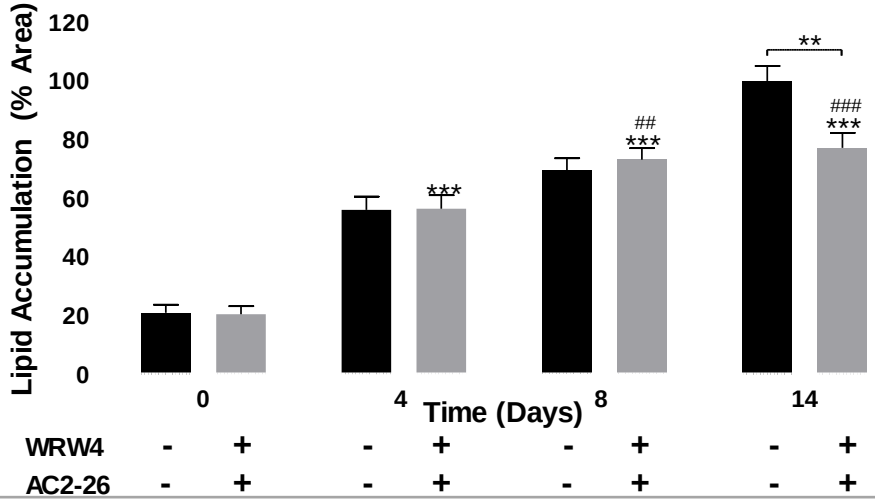


Figure 5.5: Lipid accumulation during differentiation of SGBS cells treated with FPR2/ALX antagonist; WRW4 and agonist; AC2-26. *SGBS cells were differentiated with and without 10μM WRW4 + AC2-26 and lipid droplets were stained using Oil Red O at Day 0, 4, 8 and 14. a) Visual representation of lipid droplets indicated by white arrows (magnification x10). b) Oil Red O staining was analysed using Image J. One tailed unpaired student's T-test was used to statistically analyse the data. Significant increase in lipid accumulation was observed at day 14 in comparison to day 0 ($p<0.001$, $n=6$) in WRW4 + AC2-26 differentiated cells. No significant difference in lipid accumulation was observed between vehicle control and WRW4 + AC2-26 differentiated cells between day 0 and 8. However, at Day 14, lipid accumulation was significantly less in WRW4 + AC2-26 differentiated cells compared to vehicle control ($p=0.0109$, $n=6$). Data represents as Mean ± SEM normalised to vehicle control. P value was set at $P<0.05$ and is denoted by ***= Day 0 vs, ####= 4 vs. Histogram: Black= control, Grey= treated*

Lipid accumulation in mature adipocytes differentiated with WRW4, AC2-26 or WRW4 + AC2-26, was not significantly different between each treatment, as shown in Figure 5.6.

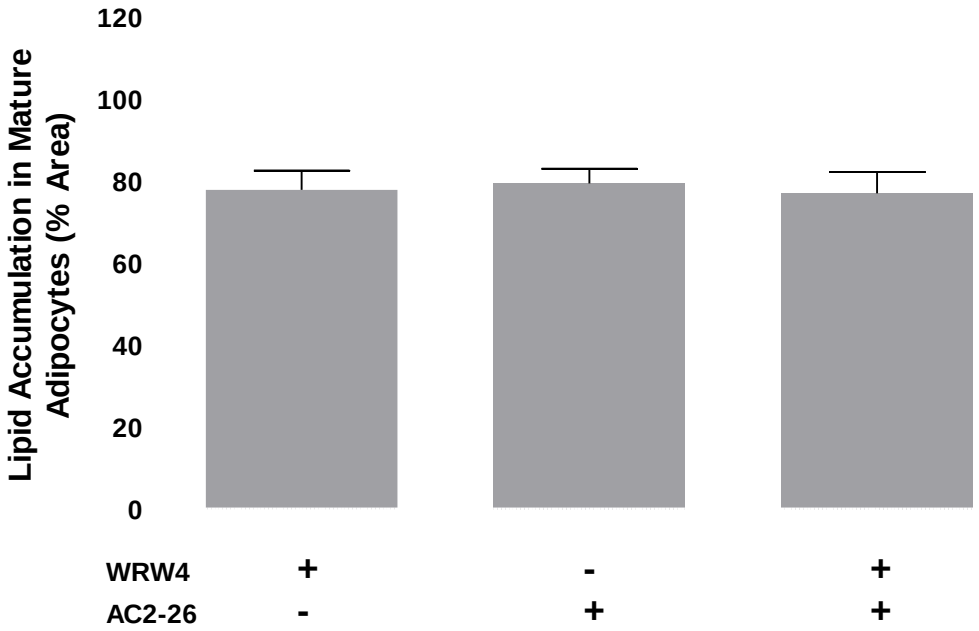
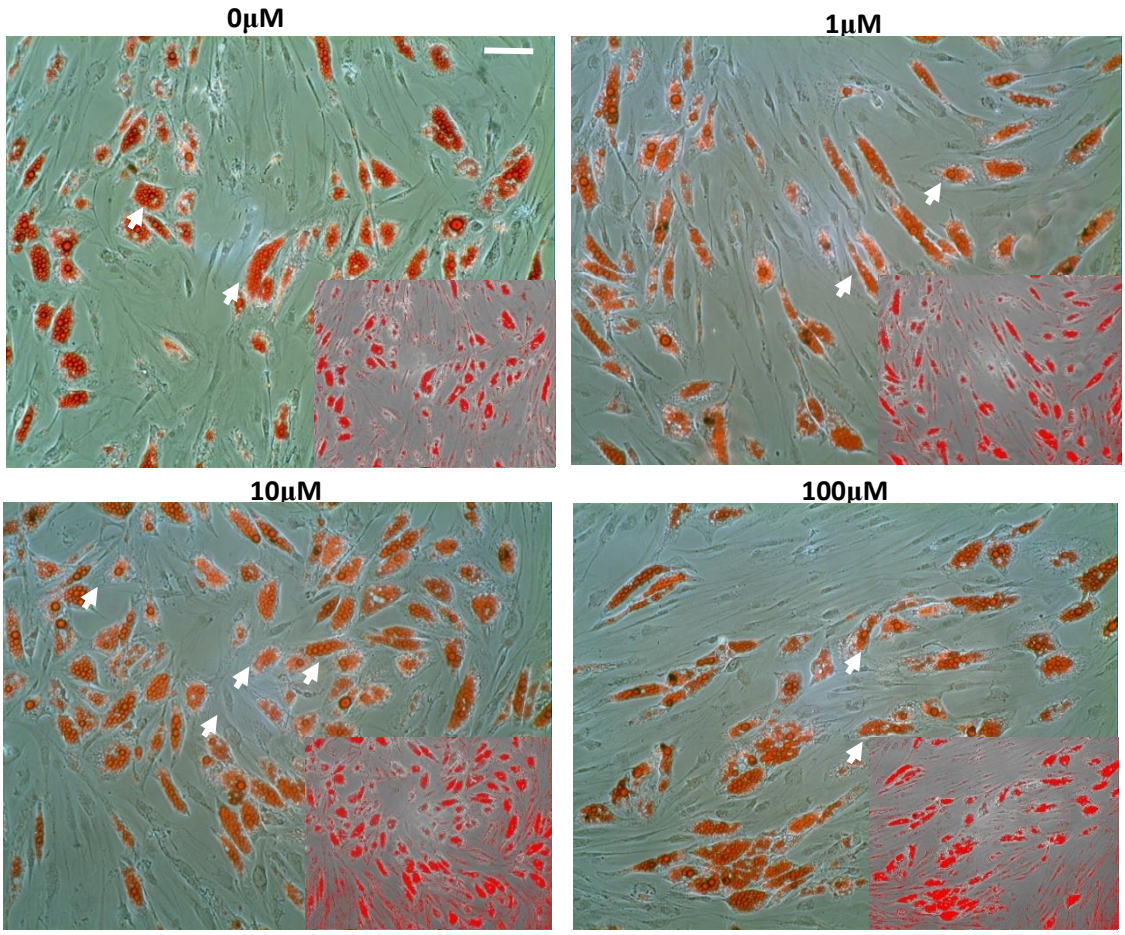


Figure 5.6: Comparison of lipid accumulation in mature adipocytes differentiated with FPR2/ALX antagonist; WRW4 and/or agonist; AC2-26. *SGBS cells were differentiated with 10μM WRW4 + AC2-26 and lipid droplets were stained using Oil Red O at Day 14. Oil Red O staining was analysed using Image J. One tailed unpaired student's T-test was used to statistically analyse the data. No Significant difference was observed between mature adipocytes differentiated with WRW4, AC2-26 or WRW4 + AC2-26. Data represents as Mean ± SEM*

To further understand the role of FPR1 in adipogenesis, a non-selective FPR antagonist; BOC-2 was used. It has a higher affinity towards FPR1, therefore at lower concentrations it binds and inhibits signalling through FPR1, however, at higher concentrations it binds and inhibits signalling through FPR2/ALX also thus, completely inhibiting ANXA1 signalling (Woloszynek et al. 2012). SGBS cells were seeded and induced to differentiate for 14 days with 0-100 μM BOC-2. At day 14, the cells were stained with ORO and assessed for lipid accumulation, as shown in Figure 5.7a. No significant difference in lipid

accumulation was observed in cells differentiated with 1μM BOC-2 at day 14, as shown in Figure 5.7b. However, lipid accumulation in cells differentiated with 10μM BOC-2 significantly increased compared to vehicle control; 120.6 ±4.3 vs 100% ±6.5 respectively, p=0.0089, n=5 at day 14. Furthermore, lipid accumulation in cells differentiated with 100μM BOC-2 was not significantly altered compared vehicle control (105.8 ±4.8, p=0.0.2364, n=5) at day 14.

a)



b)

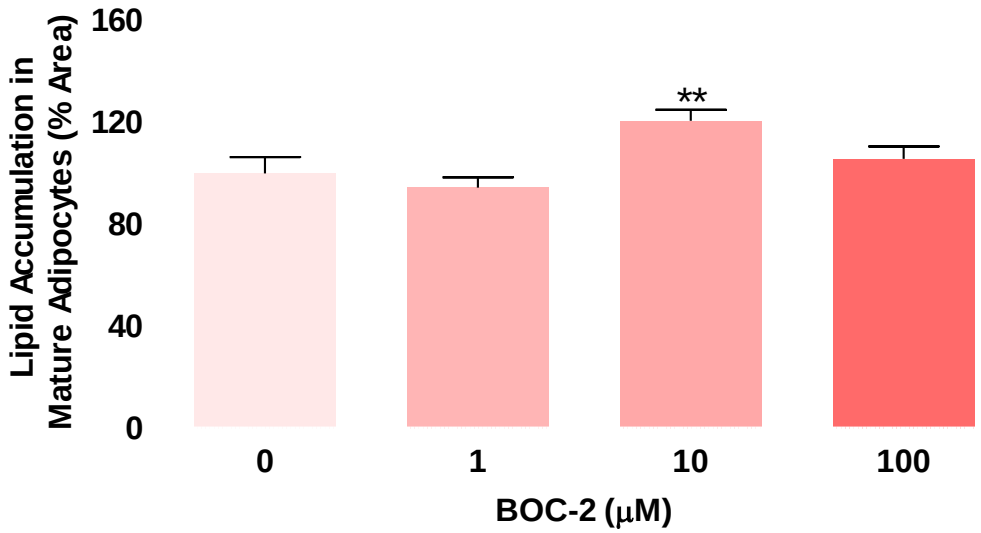


Figure 5.7: Lipid accumulation in mature adipocytes differentiated with FPR antagonist; BOC-2. *SGBS cells were differentiated with 0-100µM BOC-2 and lipid droplets were stained using Oil Red O at Day 14. Oil Red O staining was analysed using image J One tailed unpaired student's T-test was used to statistically analyse the data. a) Visual representation of lipid droplets indicated by white arrows (magnification x10). B) Mature adipocytes differentiated with 10µM accumulated significantly more lipids compared to vehicle control differentiated mature adipocytes (p=0.0089, n=5). Yet, lipid accumulation in adipocytes differentiated with 100µM did not differ compared to vehicle control differentiated mature adipocytes. Data represents as Mean ± SEM normalised to 0µM BOC-2. P value was set at P<0.05 and is denoted by * vs 0.*

5.5.2 Morphology of lipid droplets in mature adipocytes differentiated with and without FPR2/ALX receptor antagonist and/or agonist

The average number of lipid droplets in WRW4 differentiated mature adipocytes was not significantly different compared to vehicle control (96.9 ±11.5 vs 100 ±4.0, p=0.1595, n=4, respectively), as shown in Figure 5.8. Though the average size of lipid droplets was decreased, it was not significantly different (84.47 pixel^2 ±7.0 vs 100 pixel^2 ±6.9, p=0.0638, n=4, respectively), as shown in Figure 5.9. However, further analysis revealed, on average, that these cells accumulated a significantly increased number of smaller lipid

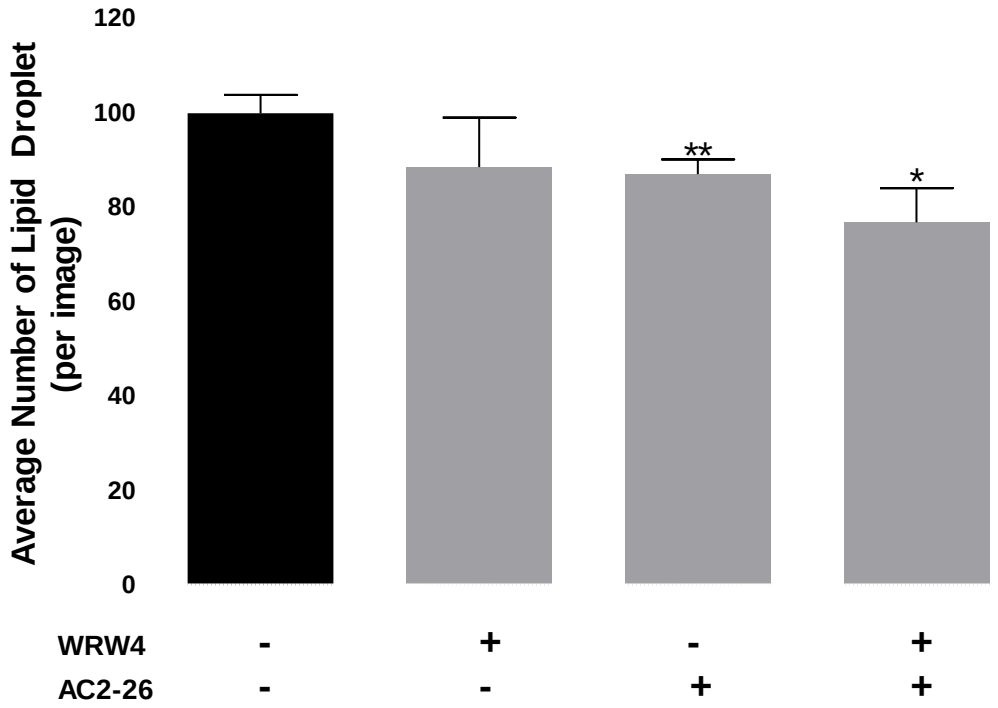


Figure 5.8: Average number of Lipid droplets in mature adipocytes differentiated with and without FPR2/ALX antagonist; WRW4 and agonist; AC2-26. *SGBS cells were differentiated with 10μM WRW4 and/or 10μM AC2-26 and lipid droplets were stained using Oil Red O at Day 14. 10 images per well of a 6 well plate was taken, and the average number of lipid droplets were analysed using Image J. One tailed unpaired student's T-test was used to statistically analyse the data. WRW4 differentiated mature adipocytes did not show a difference in the average number of lipid droplets compared to vehicle control (p=0.1595), whereas, those differentiated with AC2-26 had significantly less lipid droplets compared to vehicle control (p=0.0094, n=4). Similarly, WRW4 + AC2-26 differentiated mature adipocytes contained significantly less number of lipid droplets compared to vehicle control (p=0.0045, n=4). No statistical difference was observed between treated cells. Data represents as Mean ± SEM normalised to control. P value was set at P<0.05 and denoted by * vs control. Histogram: Black= control, Grey= treated*

droplets (between 100-1000 pixel^2) (2.3 ±0.2 vs 1.9 ± 0.1, p=0.0462, n=4) and a significantly decreased number of larger lipid droplets (1000+ pixel^2) (0.5 ±0.1 vs 0.6 ± 0.1, p=0.0200, n=4) compared to vehicle control (Figure 5.10).

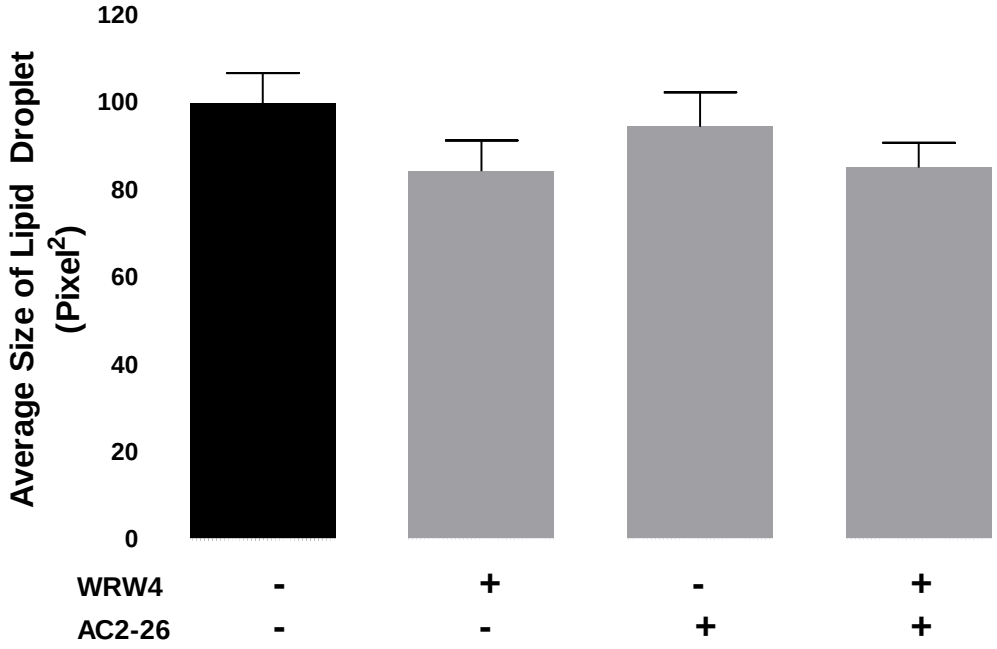


Figure 5.9: Average size of lipid droplets in mature adipocytes differentiated with or without FPR2/ALX antagonist; WRW4 and agonist; AC2-26 *SGBS cells were differentiated with 10μM WRW4 and/or 10μM AC2-26 and lipid droplets were stained using Oil Red O at Day 14. 10 images per well of a 6 well plate was taken, and the average size of lipid droplets were analysed using Image J. One tailed unpaired student's T-test was used to statistically analyse the data. WRW4 differentiated mature adipocytes showed a decrease in the average size of lipid droplets compared to vehicle control, however, it was not statically different (p=0.0638, n=4). AC2-26 differentiated mature adipocytes showed no difference in the average size of lipid droplets compared to vehicle control. Similarly, WRW4 + AC2-26 differentiated mature adipocytes showed a decrease in the average size of the lipid droplets compared to vehicle control; however, it was not statically different (p=0.0575, n=4). Data represents as Mean ± SEM normalised to control. Histogram: Black= control, Grey= treated*

However, the average number of lipid droplets in AC2-26 differentiated mature adipocytes were significantly reduced compared to vehicle control (87.1 ±3.1 vs 100 ±3.9, p=0.0094, n=4, respectively), as shown in Figure 5.8, but the average size was not different in these adipocytes compared to vehicle control (94.6 pixel^2 ±7.9 vs 100 pixel^2 ±6.9, p=0.3048, n=4, respectively), as shown in Figure5.9. Furthermore, on average, no

difference was observed in the different sizes of lipid droplets between these adipocytes and vehicle control (Figure 5.11).

Similarly, the average number of lipid droplets were significantly reduced in WRW4 + AC2-26 differentiated mature adipocytes compared to vehicle control (76.9 ±7.2 vs 100 ±3.9, p=0.0045, n=4, respectively), as shown in Figure 5.8. The average size of lipid droplets was reduced in these adipocytes compared to vehicle control, however, it was not statistically significant (85.3 ±5.7 vs 100 ±6.9, p=0.0575, n=4, respectively), as shown

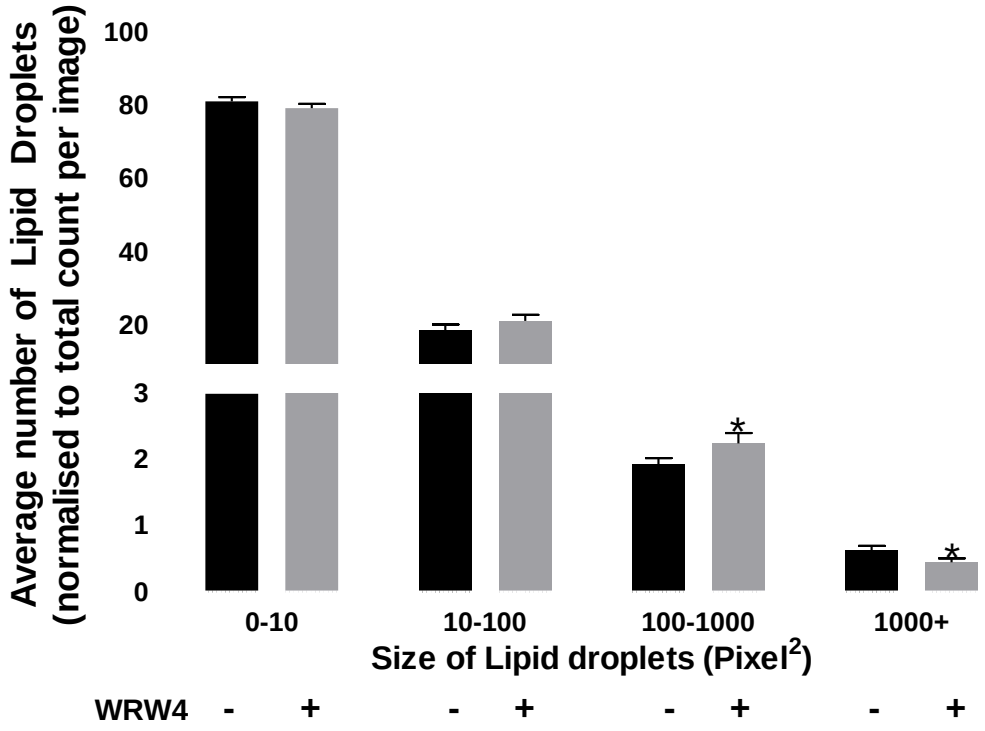


Figure 5.10: Number of lipid droplets of different sizes in mature adipocytes differentiated with FPR2/ALX antagonist; WRW4. *SGBS cells were differentiated with 10μM WRW4 and lipid droplets were stained using Oil Red O at Day 14. 10 images per well of a 6 well plate was taken, and the lipid droplet sizes were categorically analysed and normalised against the total count per image. One tailed unpaired student's T-test was used to statistically analyse the data. WRW4 differentiated mature adipocytes contained significantly increased number smaller lipid droplets (100-1000 pixel^2) compared to vehicle control (p=0.0462, n=4) and significantly decreased number of larger lipid droplets (1000+ pixel^2); p=0.0200, n=4. Data represented as Mean±SEM. P value was set at P<0.05 and is denoted by * vs control. Histogram: Black= control, Grey= treated*

in Figure 5.9. However, on average, these cells accumulated a significantly increased number of smaller lipid droplets (10-100 pixel^2) (24.2 ±1.1 vs 20.9 ±1.1, p=0.0216, n=4)

and a significantly decreased number of larger lipid droplets (1000+ pixel^2) (0.41 ±0.1 vs 0.2 ±0.1, p=0.0407, n=4) compared to vehicle control, as shown in Figure 5.12.

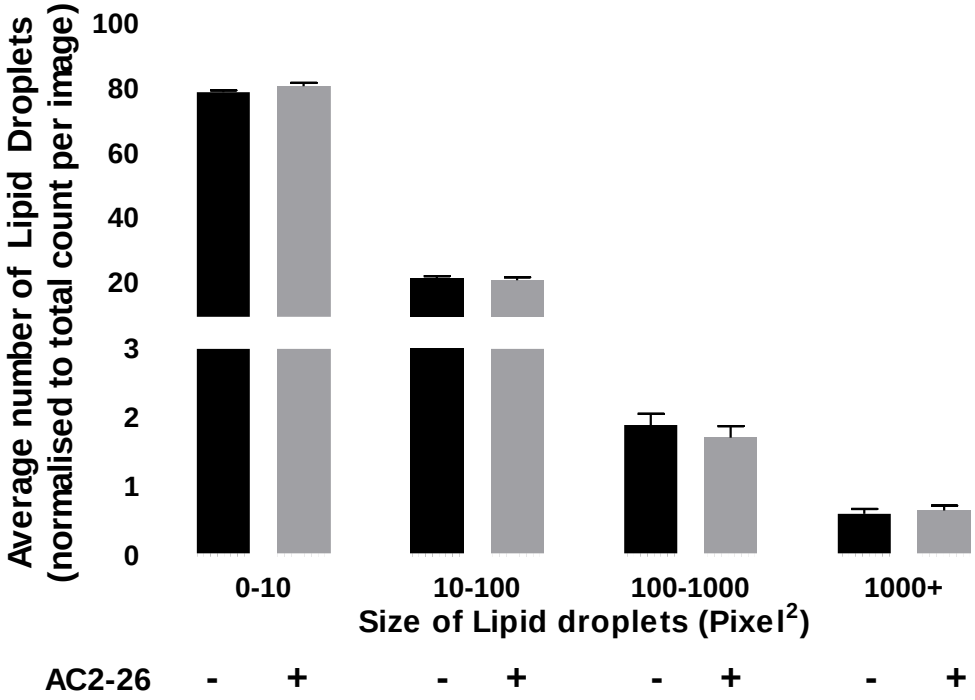


Figure 5.11: Number of lipid droplets of different sizes in mature adipocytes differentiated with FPR2/ALX agonist; AC2-26. *SGBS cells were differentiated with 10μM AC2-26 and lipid droplets were stained using Oil Red O at Day 14. 10 images per well of a 6 well plate was taken, and the lipid droplet sizes were categorically analysed and normalised against the total count per image. One tailed unpaired student's T-test was used to statistically analyse the data. No difference in the size of lipid droplets was observed between AC2-26 differentiated mature adipocytes and vehicle control (n=4). Data represented as Mean±SEM. Histogram: Black= control, Grey= treated*

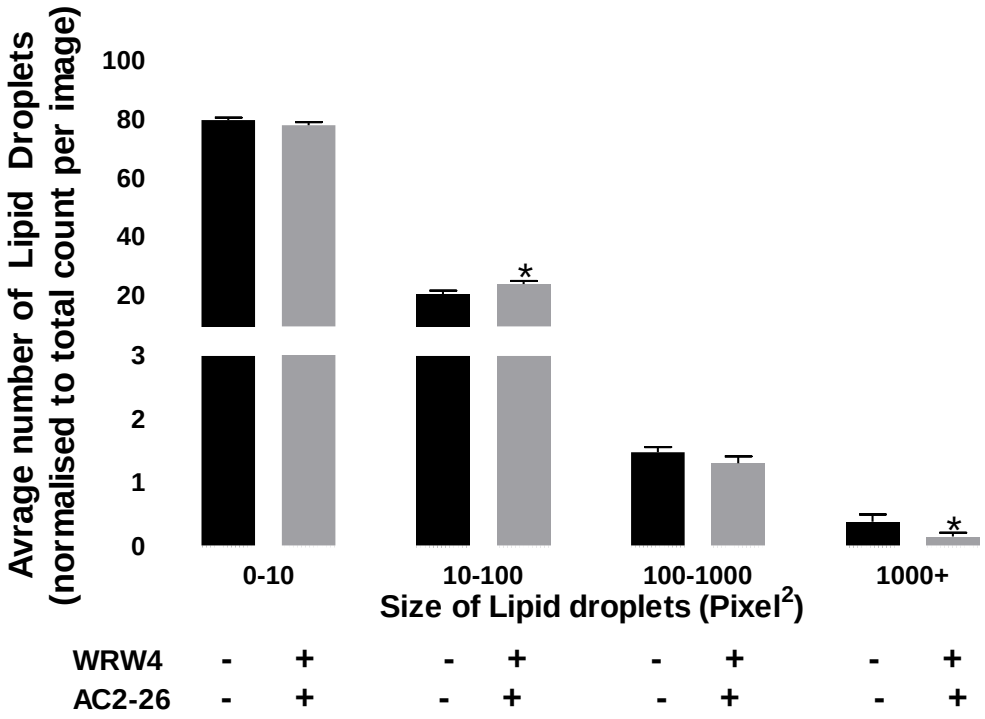


Figure 5.12: Average number of lipid droplets of different sizes in mature adipocytes differentiated with FPR2/ALX antagonist; WRW4 and agonist; AC2-26. *SGBS cells were differentiated with 10μM WRW4 and 10μM AC2-26 and lipid droplets were stained using Oil Red O at Day 14. 10 images per well of a 6 well plate was taken, and the lipid droplet sizes were categorically analysed and normalised against the total count per image. One tailed unpaired student's T-test was used to statistically analyse the data. WRW4 + AC2-26 differentiated mature adipocytes contained significantly increased number smaller lipid droplets (10-100 pixel^2) compared to vehicle control (p=0.0216, n=4) and significantly decreased number of larger lipid droplets (1000+ pixel^2); p=0.0407, n=4. Data represented as Mean±SEM. P value was set at P<0.05 and is denoted by * vs control. Histogram: Black= control, Grey= treated*

Moreover, on average, AC2-26 only differentiation of mature adipocytes resulted in a significantly increased number of smaller lipid droplets (0-10 pixel^2) compared to WRW4 + AC2-26 differentiated mature adipocytes; p=0.0436. Whereas, WRW4 + AC2-26 differentiated mature adipocytes showed increased number of 10-100 pixel^2 lipid droplets compared to AC2-26 only differentiated mature adipocytes (p= 0.0165). The average number of lipid droplets between 100-1000+ were significantly altered in all conditions, as shown in Figure 5.13. AC2-26 only differentiated mature adipocytes and WRW4 + AC2-26 differentiated mature adipocytes accumulated significantly less 100-1000 pixel^2

lipid droplets (p=0.0143 and p<0.0001, n=4, respectively) compared to WRW4 only differentiated mature adipocytes. Similarly, WRW4 + AC2-26 differentiated mature adipocytes contained significantly fewer 100-1000 pixel^2 lipid droplets compared to AC2-26 only differentiated mature adipocytes; p=0.0277, n=4. AC2-26 only differentiated adipocytes contained significantly increased number of larger lipid droplets (1000+ pixel^2) compared to WRW4 only differentiated adipocytes (p=0.0169, n=4) and WRW4 + AC2-26 differentiated adipocytes (p<0.0001, n=4). Whereas, WRW4 + AC2-26 differentiated mature adipocytes contained significantly fewer large lipid droplets

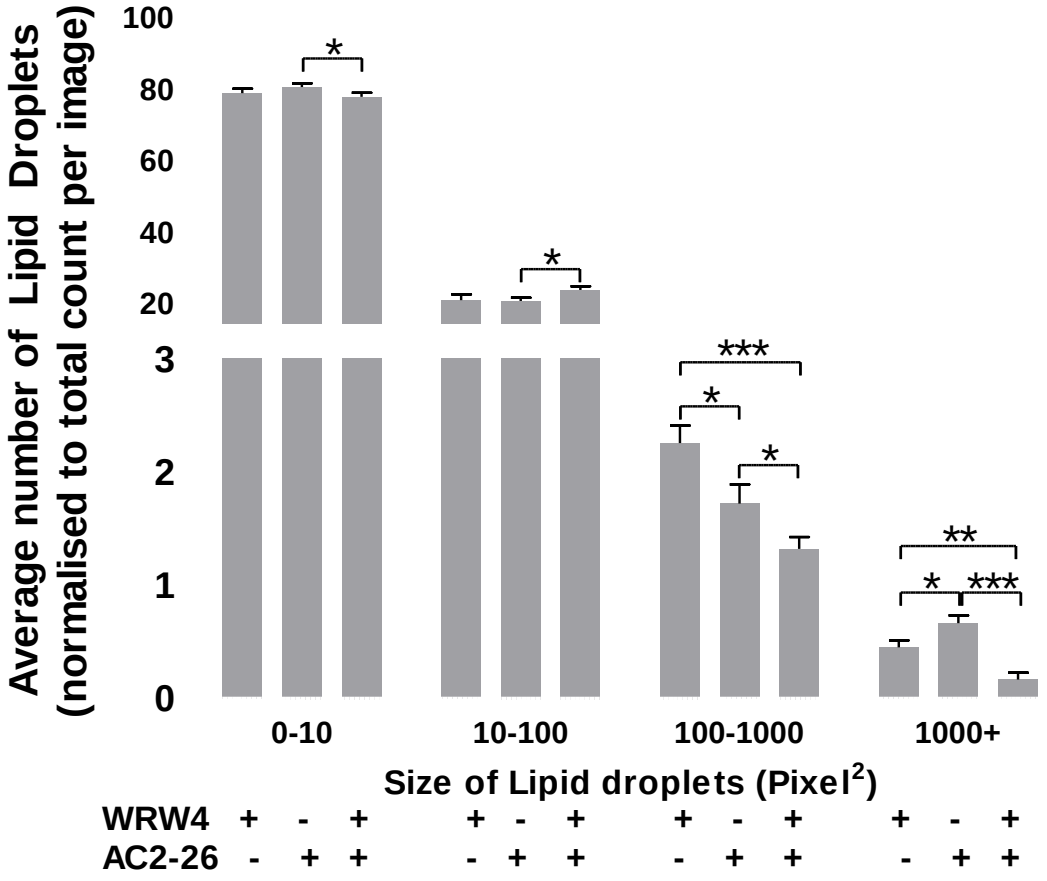


Figure 5.13: Comparison of average number of lipid droplets of different sizes in mature adipocytes differentiated with or without FPR2/ALX antagonist; WRW4 and/or agonist; AC2-26. *SGBS cells were differentiated with 10μM WRW4 and/or 10μM AC2-26 and lipid droplets were stained using Oil Red O at Day 14. 10 images per well of a 6 well plate was taken, and the lipid droplet sizes were categorically analysed using Image J and normalised against the total count per image. One tailed unpaired student's T-test was used to statistically analyse the data. Data represented as Mean±SEM. P value was set at P<0.05 and is denoted by *.*

(1000+ pixel^2) compared to WRW4 only differentiated mature adipocytes (p=0.0015, n=4).

5.5.3 Cytotoxicity of antagonist and agonist compounds in mature adipocytes

To ensure the results observed were a result of the receptor antagonist and agonist and not due to cellular cytotoxicity of the compounds, cell viability was assessed using the AlamarBlue cell viability assay, as described in section 5.4.3.

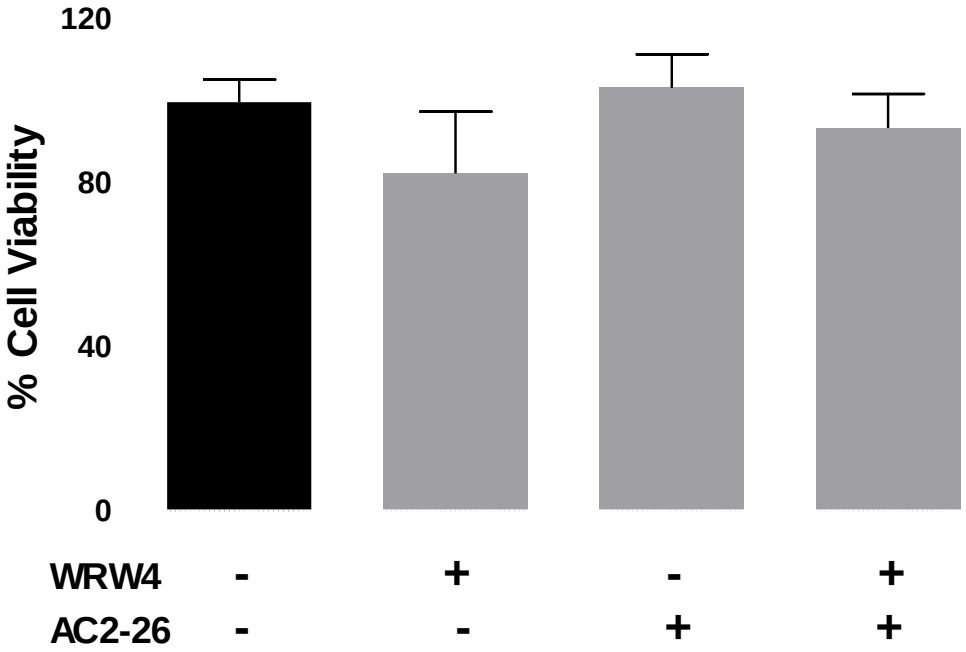


Figure 5.14: Cell viability in mature adipocytes differentiated with and without FPR2/ALX antagonist; WRW4 and/or agonist: AC2-26. *SGBS cells were differentiated with 10μM WRW4 and/or 10μM AC2-26 over 14 days to reach a mature adipocyte phenotype. On day 14, the cells were incubated with 10% AlamarBlue for 4 hours at 37C. The absorbance was measured using a spectrophotometer at 562nm and 600nm as a reference wavelength. Cell viability was normalised against control and is presented as %. One tailed unpaired student's T-test was used to statistically analyse the data. No significant difference in % cell viability is observed between vehicle control and WRW4 differentiated mature adipocytes (p=0.1385, n=4), vehicle control and AC2-26 differentiated mature adipocytes (p=0.3622, n=4) and vehicle control and WRW4 + AC2-26 differentiated mature adipocytes (0.2688, n=4). Similarly, no difference in % cell viability as observed between treated mature adipocytes. Data represents as Mean ± SEM normalised to control. Histogram: Black= control, Grey= treated*

SGBS cells were seeded and induced to differentiate for 14 days with 10 µM WRW4 and/or 10 µM AC2-26 or vehicle control and cellular viability was assessed, as shown in Figure 5.14. No significant difference in percentage of cell viability was observed between vehicle control and WRW4 differentiated mature adipocytes; 100% ±5.6 vs 82.7% ±15.1, p=0.01385, n=4, respectively. Similarly, no difference in percentage of cell viability was observed between vehicle control and AC2-26 differentiated mature adipocytes; 100% ±5.6 vs 103.5% ±8.3, p=0.3622, n=4, respectively. Furthermore, no difference in percentage of cell viability was observed between vehicle control and WRW4 + AC2-26 differentiated mature adipocytes; 100% ±5.6 vs 93.6% ±8.3, p=0.2688, n=4, respectively. No difference in percentage of cell viability was observed between treatment differentiated mature adipocytes.

5.5.4 Intracellular signalling pathway activation in Mature Adipocytes differentiated with and without FPR2/ALX receptor antagonist and/or agonist

To investigate whether endogenous AC2-26 binds to FPR1 upon FPR2/ALX antagonist and agonist treatment, intracellular signalling pathways of FPR1 and FPR2/ALX were analysed. ANXA1/AC2-26 binding to and activating FPR2/ALX is thought to activate ERK1/2 MAPK whereas, AC2-26 binding to and activating FPR1 is thought to activate p38 mitogen activated protein kinase (p38 MAPK). SGBS cells were seeded and induced to differentiate for 14 days with 10 µM WRW4 and/or 10 µM AC2-26 or vehicle control and phosphorylation of ERK1/2 MAPK and p38 MAPK were analysed.

The percentage of phosphorylated ERK1/2 MAPK (%p-ERK1/2) was slightly decreased in mature adipocytes differentiated with WRW4 and/or AC2-26, however, it was not statistically significant compared to vehicle control, as shown in Figure 5.15. %p-ERK1/2

was decreased to 92.8% ±14.8 in WRW4 differentiated mature adipocytes compared to vehicle control; 100% ±8.3, p=0.3409, n=5. Similarly, %p-ERK1/2 was decreased to 95.2% ±13.9 in AC2-26 differentiated mature adipocytes compared to vehicle control; 100% ±8.3, p=0.3879, n=5. Furthermore, %p-ERK1/2 was decreased to 97.4% ±14.4 in WRW4 + AC2-26 differentiated mature adipocytes compared to vehicle control; 100% ±8.3, p=0.4369.16, n=5. Consequently, no difference was found between differently treated mature adipocytes.

The percentage of phosphorylated p38 MAPK (%p-p38) was significantly decreased in WRW4 differentiated mature adipocytes (64.8% ±15.1 vs 100% ±6.3, p=0.0317, n=5), in AC2-26 differentiated mature adipocytes (71.4% ±4.7 vs 100% ±6.3, p=0.0024, n=6) and in WRW4 + AC2-26 differentiated mature adipocytes (67.1% ±15.1 vs 100%±6.3, p=0.0394, n=5) compared to vehicle control, as shown in Figure 5.16. However, no significant difference was observed between differently treated mature adipocytes.

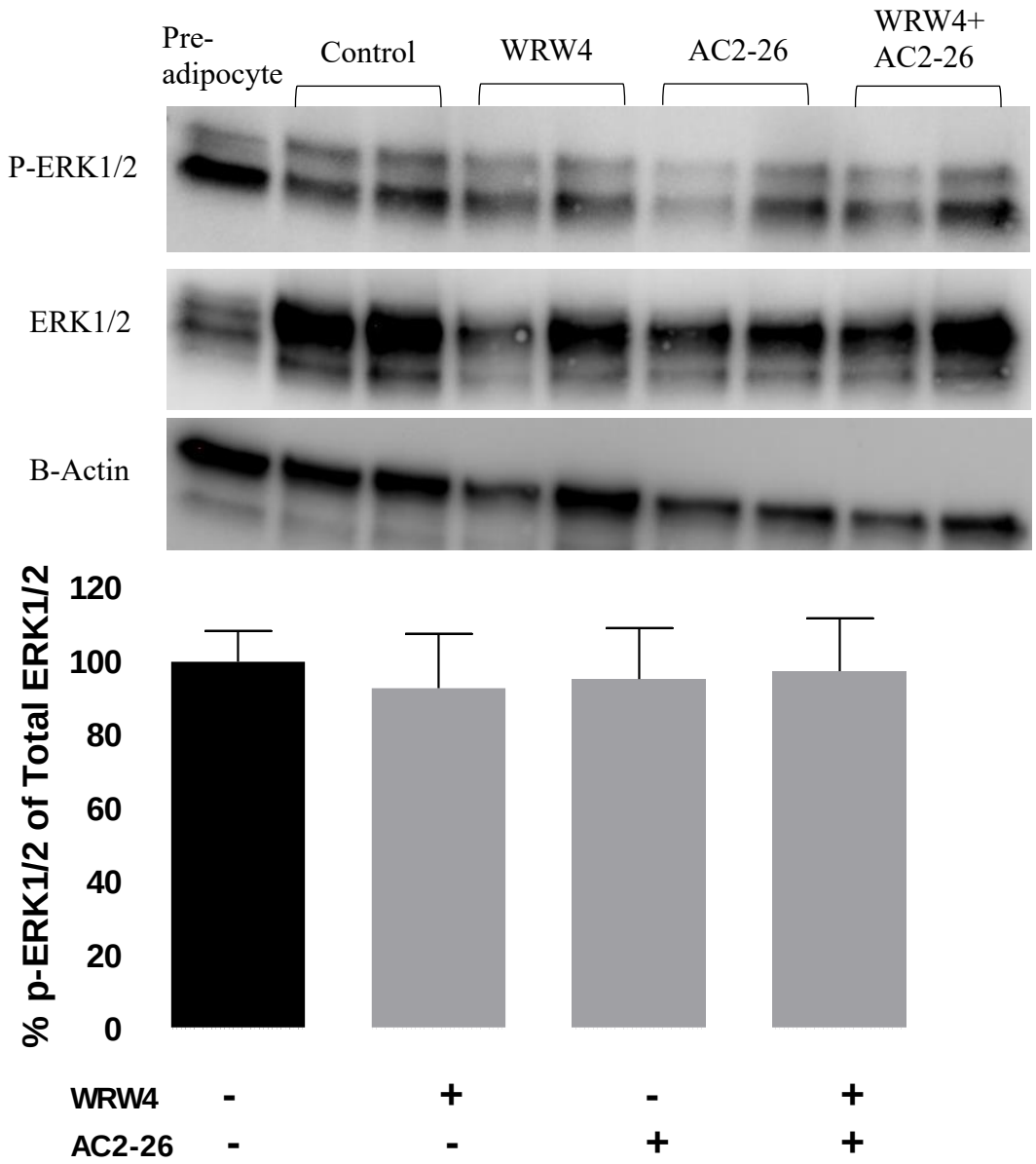


Figure 5.15: ERK1/2 MAPK activation in mature adipocytes differentiated with and without FPR2/ALX antagonist; WRW4 and/or agonist; AC2-26. *SGBS cells were differentiated with 10μM WRW4 and 10μM AC2-26 over 14 days to reach a mature adipocyte phenotype. On day 14, the cells were lysed using RIPA buffer and intracellular proteins were extracted and the concentration determined using BCA assay. 40μg of protein per well was loaded into SDS gels along with the sample buffer and run for 90 minutes at 150V. The separated proteins were transferred onto a PVDF membrane. The membrane was incubated in 5% milk for an hour, washed with TBST x3 and incubated with 1:2000 primary anti-p-ERK1/2 antibody overnight. The membrane was washed with TBST x3 and incubated with 1:10,000 secondary anti-IgG antibody for 2 hours. It was washed with TBST x3 and visualised using the SuperSignal west femto kit. The antibodies were stripped the membrane using stripping buffer and the processes was repeated for ERK1/2 and βActin. The density of the bands was analysed using Image J. One tailed unpaired student's T-test was used to statistically analyse the data. % phosphorylated p-ERK MAPK was slightly decreased in mature adipocytes differentiated with WRW4 and/or AC2-26, however, it should no statistical difference; WRW4 differentiated mature adipocytes; p=0.3192, n=5, AC2-26 differentiated mature adipocytes; p=0.3700, n=5, and WRW4 + AC2-26 differentiated mature adipocytes; p=0.4216, n=4, compared to vehicle control. Data presented as Mean±SEM normalised to control. Histogram: Black= control, Grey= treated*
ERK MAPK= Extracellular signal regulated kinase and MAPK=Mitogen activated protein kinase

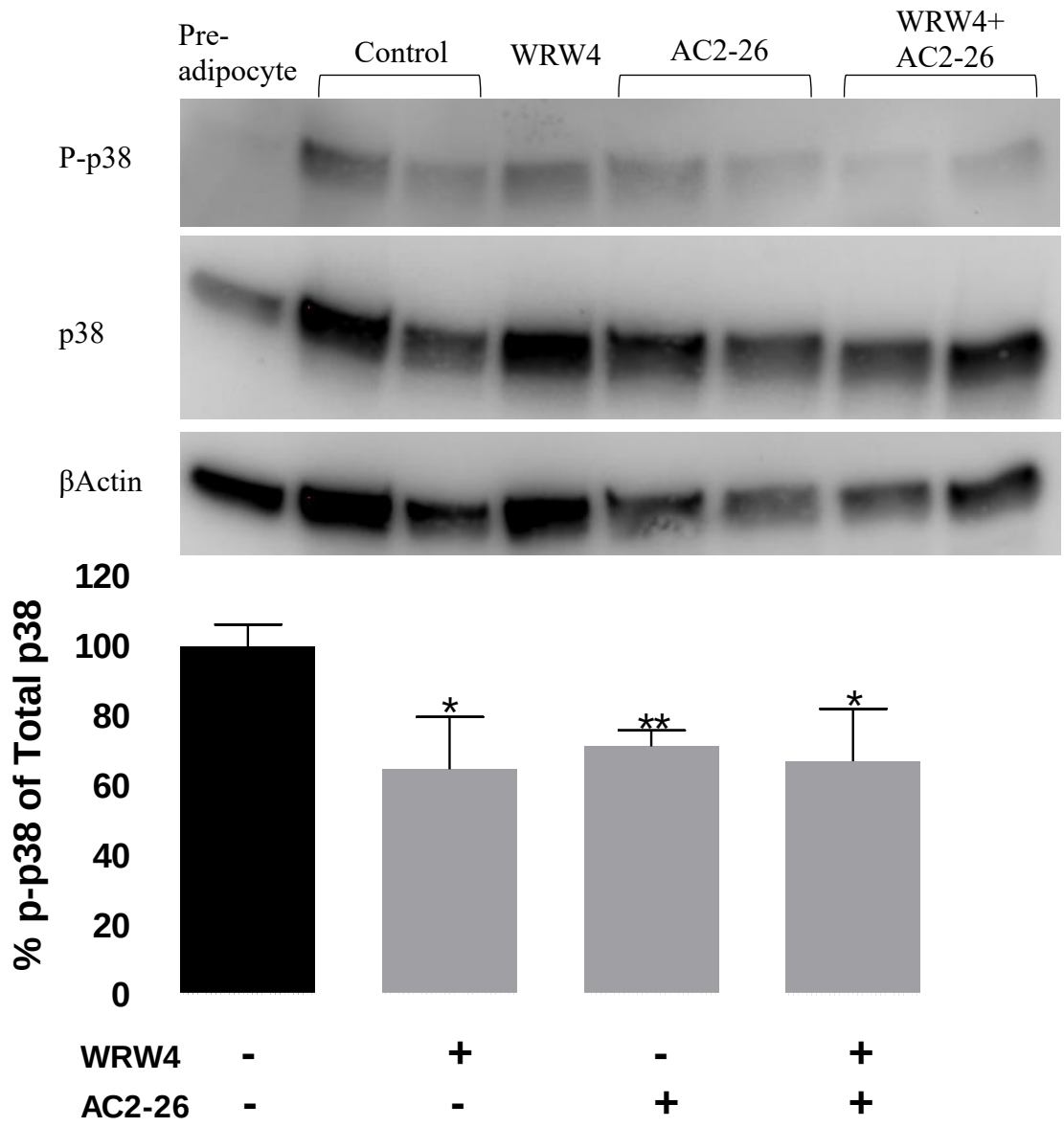


Figure 4.16: p38 MAPK activation in mature adipocytes differentiated with and without FPR2/ALX antagonist; WRW4 and/or agonist; AC2-26. *SGBS cells were differentiated with 10μM WRW4 and 10μM AC2-26 over 14 days to reach a mature adipocyte phenotype. On day 14, the cells were lysed using RIPA buffer and intracellular proteins were extracted and the concentration determined using BCA assay. 40μg of protein per well was loaded into SDS gels along with the sample buffer and run for 90 minutes at 150V. The separated proteins were transferred onto a PVDF membrane. The membrane was incubated in 5% milk for an hour, washed with TBST x3 and incubated with 1:1000 primary anti-p-p38antibody overnight. The membrane was washed with TBST x3 and incubated with 1:10,000 secondary anti-IgG antibody for 2 hours. It was washed with TBST x3 and visualised using the SuperSignal west femto kit. The antibodies were stripped the membrane using stripping buffer and the processes was repeated for p38 and βActin. The density of the bands was analysed using Image J. One tailed unpaired student's T-test was used to statistically analyse the data. % phosphorylated p-38 MAPK was significantly decreased in WRW4 differentiated mature adipocytes (p=0.0317, n=5), in AC2-26 differentiated mature adipocytes (p=0.0024, n=6) and in WRW4 + AC2-26 differentiated mature adipocytes (p=0.0394, n=5) compared to vehicle control. However, no significant difference was found between treated cells. Data presented as Mean±SEM normalised to control. * vs control. Histogram: Black= control, Grey= treated*
p38 MAPK= p38 mitogen activated protein kinase

5.5.5 *De novo* lipogenesis in mature adipocytes differentiated with and without FPR2/ALX receptor antagonist and/or agonist

Active p38 MAPK phosphorylates several transcription factors involved in lipogenesis including transcriptional factors and enzymes through *de-novo* lipogenesis. Therefore, transcriptional factors and enzymes involved in de-novo lipogenesis were assessed.

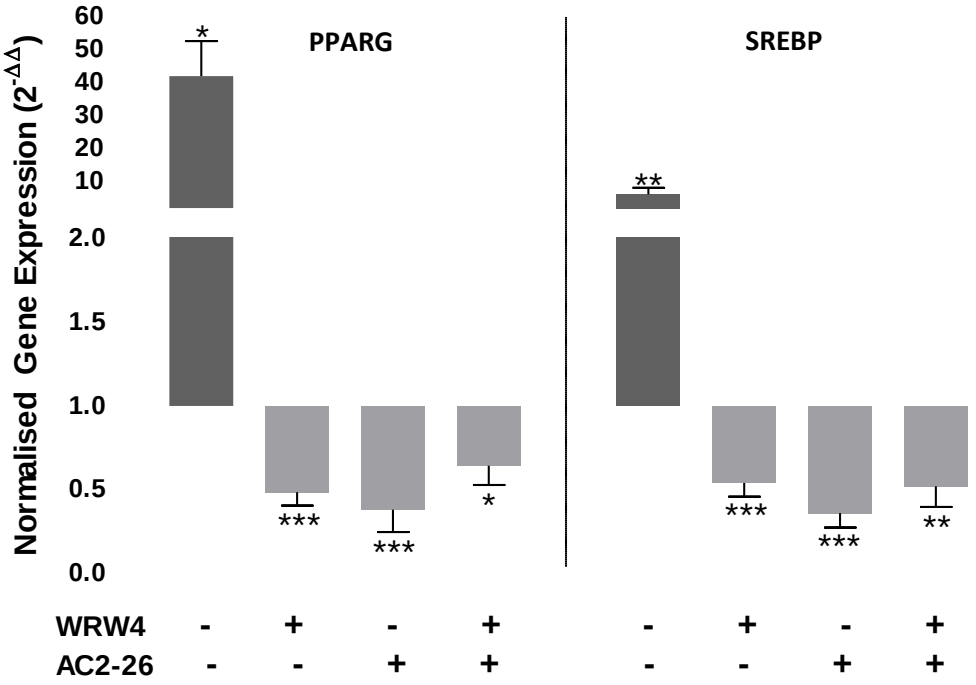


Figure 5.17: mRNA expression of transcription factors involved in lipogenesis in mature adipocytes differentiated with and without FPR2/ALX antagonist; WRW4 and/or agonist: AC2-26. *SGBS cells were differentiated with 10μM WRW4 and/or 10μM AC2-26 over 14 days to reach a mature adipocyte phenotype. On day 14, mRNA was extracted using Trisure and cDNA was synthesised. 80ng of cDNA was used in SYBR green mediated quantitative PCR. Ct values were obtained and used to calculate normalised gene expression using the Livak $2^{-\Delta\Delta CT}$ equation (as described in Chapter 3). One tailed unpaired student's T-test was used to statistically analyse the data. mRNA expression of PPARγ (PPARG) was significantly upregulated in mature adipocytes compared to preadipocytes (p=0.0098, n=7) and significantly downregulated in WRW4 differentiated mature adipocytes (p<0.0001, n=5), in AC2-26 differentiated mature adipocytes (p=0.0008, n=5) and in WRW4 + AC2-26 differentiated mature adipocytes (0.0103, n=4) compared to vehicle control. Similarly, mRNA expression of SREB (SREBP) was significantly upregulated in mature adipocytes compared to preadipocytes (p=0.0467, n=8) and significantly downregulated in WRW4 differentiated mature adipocytes (p=0.0002, n=4), in AC2-26 differentiated mature adipocytes (p<0.0001, n=4) and in WRW4 + AC2-26 differentiated mature adipocytes (p=0.0037, n=4) compared to vehicle control. Preadipocytes and vehicle control normalised to 1. Data presented as Mean±SEM. P value was set at P<0.05 and is denoted by * vs 1. Histogram: Dark Grey= control, Grey= treated. Housekeeping gene:18s RNA*
PPARγ= Peroxisome proliferator activated receptor γ and SREBP= Sterol regulatory element-binding protein

SGBS cells were seeded and induced to differentiate for 14 days with 10μM WRW4 and/or 10μM AC2-26 and mRNA expression levels were studied.

As expected, mRNA expression of transcriptional factors; *PPARG* and *SREBP* were significantly upregulated in mature adipocytes compared to preadipocytes; 41.8 ±10.6-fold, p=0.0098, n=7 and 6.1 ±1.9-fold, p=0.0467, n=8 vs 1, n=8 respectively, as shown in Figure 5.17. Similarly, mRNA expression levels of lipogenic enzymes were significantly upregulated in mature adipocytes in comparison to preadipocytes, as shown in Figure 5.18. Gene expression levels of *ACC, FAS* and *GPAT* were significantly upregulated by 16.2 ±2.6-fold, p=0.0013, n=8, 70.2 ±20.5-fold, p=0.0173, n=7 and 80.0 ±34.7-fold, p=0.0424, n=5 respectively, compared to preadipocytes (1, n=8).

Interestingly, mRNA expression of PPARγ (*PPARG*) and SREBP (*SREBP*) were significantly downregulated in WRW4 differentiated mature adipocytes (0.49 ±0.08-fold, p<0.0001, n=5 and 0.54 ±0.08-fold, p=0.0002, n=4, respectively), in AC2-26 differentiated mature adipocytes (0.38 ±0.1-fold, p=0.0008, n=5 and 0.36 ±0.1-fold, p<0.0001, n=4, respectively) and in WRW4 + AC2-26 differentiated mature adipocytes (0.64 ±0.1, p=0.0103, n=4 and 0.52 ±0.1, p=0.0037, n=4, respectively), compared to vehicle control (1, n=5), as shown in Figure 5.17.

Similarly, mRNA expressions of lipogenesis enzymes were dysregulated, as shown in Figure 5.18. mRNA expression of acetyl CoA carboxylase (*ACC*) was significantly downregulated in WRW4 differentiated mature adipocytes (0.73 ±0.1, p=0.0154, n=4), in AC2-26 differentiated mature adipocytes (0.20 ±0.1, p<0.0001, n=5) and in WRW4 + AC2-26 differentiated mature adipocytes (0.62 ±0.1, p=0.0213, n=6) compared to vehicle control (1, n=6). mRNA expression of fatty acid synthase (*FASN*) was significantly

downregulated in WRW4 differentiated mature adipocytes (0.62 ±0.1, p=0.0081, n=5) and in WRW4 + AC2-26 differentiated mature adipocytes (0.80 ±0.1, p=0.0071, n=5) compared to vehicle control (1, n=5). However, no significant difference was observed in the mRNA expression of *FAS* in AC2-26 differentiated mature adipocytes (1.10 ±0.2, p=0.3540, n=4) compared to vehicle control (1, n=4). mRNA expression of glycerol-3-phosphate acyltransferase (*GPAM*) was significantly upregulated in WRW4 differentiated mature adipocytes (2.69 ±0.6, p=0.0270, n=7), significantly downregulated in AC2-26 differentiated mature adipocytes (0.69 ±0.1, p=0.0031, n=5) and in WRW4 + AC2-26 differentiated mature adipocytes (0.67 ±0.1, p=0.0144, n=4) compared to vehicle control (1, n=7).

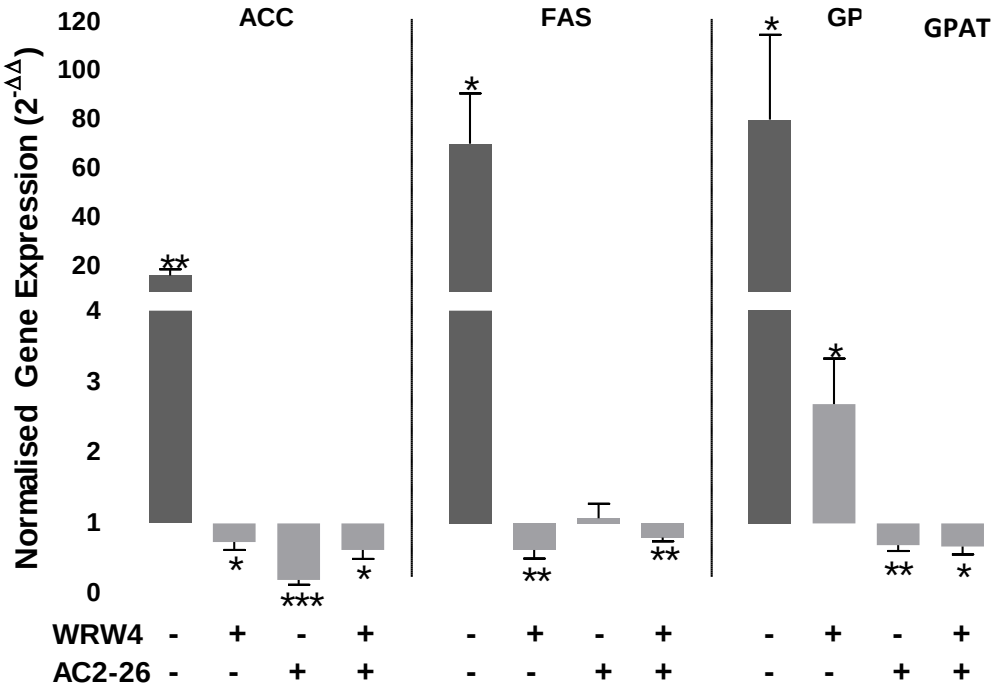
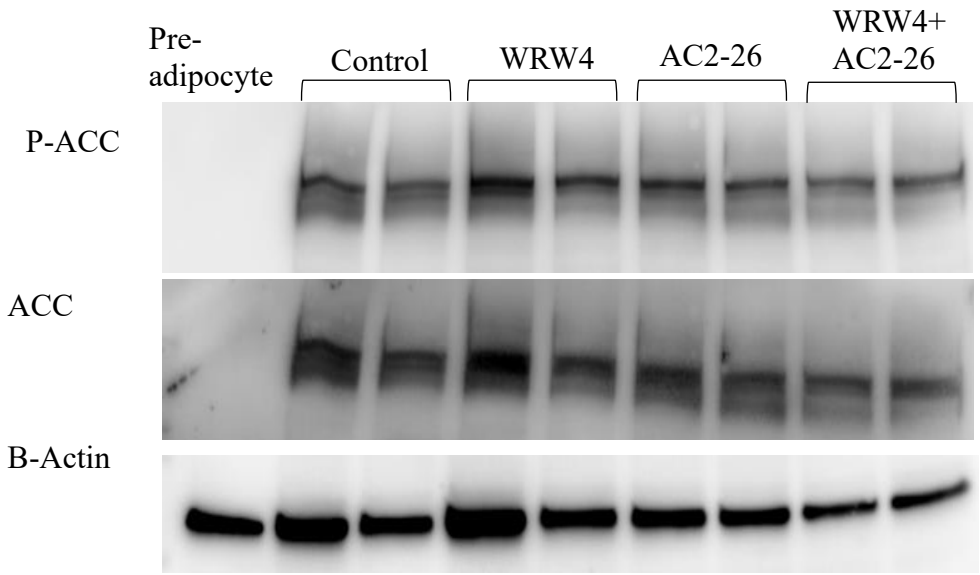


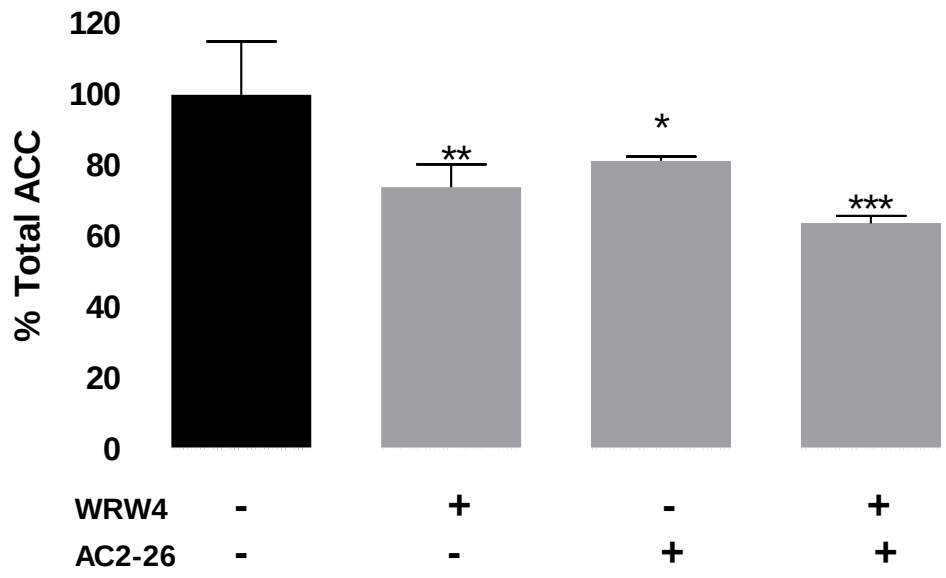
Figure 5.18: mRNA expression of enzymes involved in lipogenesis in mature adipocytes differentiated with and without FPR2/ALX antagonist; WRW4 and/or agonist: AC2-26. *SGBS cells were differentiated with 10μM WRW4 and/or 10μM AC2-26 over 14 days to reach a mature adipocyte phenotype. On day 14, mRNA was extracted using Trisure and cDNA was synthesised. 80ng of cDNA was used in SYBR green mediated quantitative PCR. Ct values were obtained and used to calculate normalised gene expression using the Livak $2^{-\Delta\Delta CT}$ equation (as described in Chapter 3). One tailed unpaired student's T-test was used to statistically analyse the data. mRNA expression of ACC (ACC) was significantly upregulated in mature adipocytes compared to preadipocytes (p=0.0013, n=8) and significantly downregulated in WRW4 differentiated mature adipocytes (p=0.0154, n=4), in AC2-26 differentiated mature adipocytes (p<0.0001, n=5) and in WRW4 + AC2-26 differentiated mature adipocytes (0.0213, n=6) compared to vehicle control. Similarly, mRNA expression of FAS (FASN) was significantly upregulated in mature adipocytes compared to preadipocytes (p=0.0173, n=7) and significantly downregulated in WRW4 differentiated mature adipocytes (p=0.0081, n=5) and in WRW4 + AC2-26 differentiated mature adipocytes (p=0.0071, n=5) compared to vehicle control. However, no significant difference was observed in the mRNA expression of FASN in AC2-26 differentiated mature adipocytes compared to vehicle control (p=0.3540, n=4). mRNA expression of GPAT (GPAM) was significantly upregulated in mature adipocytes compared to preadipocytes (p=0.0424, n=5) and in WRW4 differentiated mature adipocytes (p=0.0270, n=7), whereas it was significantly downregulated in AC2-26 differentiated mature adipocytes (p=0.0031, n=5) and in WRW4 + AC2-26 differentiated mature adipocytes (p=0.0144, n=4) compared to vehicle control. Preadipocytes and vehicle control normalised to 1. Data presented as Mean±SEM. P value was set at P<0.05 and is denoted by * vs 1. Dark Grey= control, Grey= treated. Housekeeping gene:18s RNA*
ACC= acetyl CoA carboxylase, FAS= Fatty acid synthase and GPAT= Glycerol-3-phoshphate acyltransferase 1

A similar pattern was observed at the proteomic level for ACC. The total percentage of ACC was significantly reduced in WRW4 differentiated mature adipocytes (70.2% ±9.5, p=0.00343, n=3), in AC2-26 differentiated mature adipocytes (86.2% ±4.9, p=0.0146, n=4) and in WRW4 + AC2-26 mature adipocytes (63.8% ±2.1, p<0.0001, n=3) compared to vehicle control (100% ±15, n=4, as shown in Figure 5.19. Furthermore, percentage phosphorylated ACC (%pACC) was also significantly decreased in WRW4 differentiated mature adipocytes (70.2% ±9.5, p=0.0343, n=5), in AC2-26 differentiated mature adipocytes (59.2% ±10.2, p=0.0130, n=6) and in WRW4 + AC2-26 differentiated mature adipocytes (58.8% ±13.1, p=0.0265, n=6) compared to vehicle control (100% ±10, n=4), as shown in Figure 5.19.

a)


Pre-adipocyte
Control
WRW4
AC2-26
WRW4+ AC2-26
P-ACC
ACC
B-Actin

b)


% Total ACC
120
100
80
60
40
20
0
**
*
***
WRW4 - + - +
AC2-26 - - + +

c)

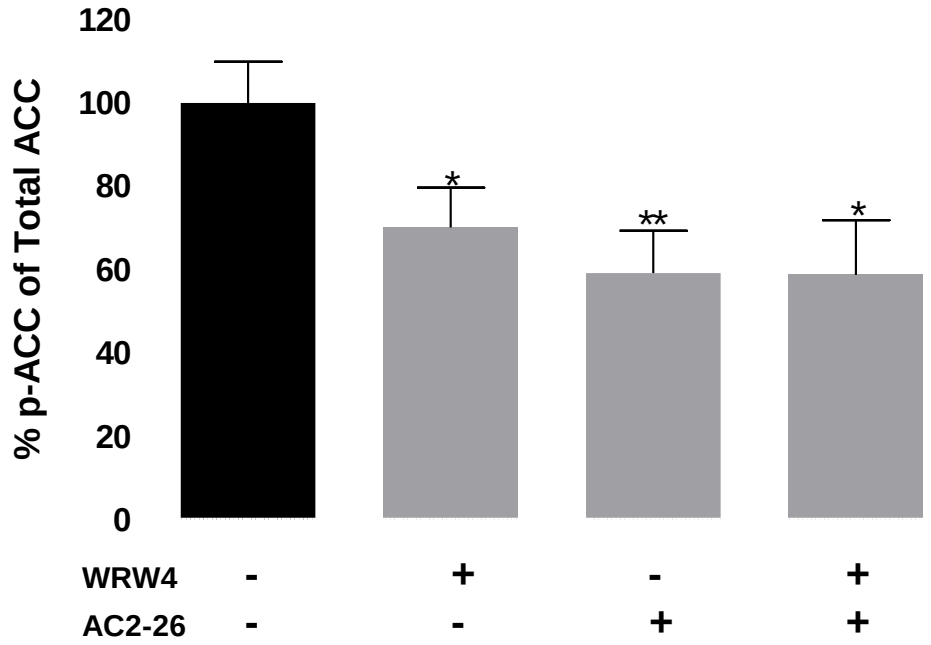


Figure 5.19: % protein expression of ACC in mature adipocytes differentiated with and without FPR2/ALX antagonist; WRW4 and/or agonist; AC2-26. *SGBS cells were differentiated with 10μM WRW4 and 10μM AC2-26 over 14 days to reach a mature adipocyte phenotype. On day 14, the cells were lysed using RIPA buffer and intracellular proteins were extracted and the concentration determined using BCA assay. 60μg of protein per well was loaded into SDS gels along with the sample buffer and run for 90 minutes at 150V. The separated proteins were transferred onto a PVDF membrane. The membrane was incubated in 5% milk for an hour, washed with TBST x3 and incubated with 2:1000 primary anti-p-ACC antibody overnight. The membrane was washed with TBST x3 and incubated with 1:10,000 secondary anti-IgG antibody for 2 hours. It was washed with TBST x3 and visualised using the SuperSignal west femto kit. The antibodies were stripped the membrane using stripping buffer and the processes was repeated for total ACC and βActin. The density of the bands was analysed using Image J. One tailed unpaired student's T-test was used to statistically analyse the data. b) % of total protein was significantly decreased in WRW4 differentiated mature adipocytes (p=0.0024, n=3), in AC2-26 differentiated mature adipocytes (p=0.0146, n=4) and in WRW4 + AC2-26 differentiated mature adipocytes (o<0.0001, n=3), compared to vehicle control. c) % phosphorylated p-ACC was significantly decreased in mature adipocytes differentiated with WRW4 and/or AC2-26, however, it should no statistical difference; WRW4 differentiated mature adipocytes (p=0.0343, n=5), in AC2-26 differentiated mature adipocytes (0.0130, n=6) and in WRW4 + AC2-26 differentiated mature adipocytes (p=0.0265, n=6) compared to vehicle control. Data presented as Mean±SEM. P value was set at P<0.05 and is denoted by * vs control. Black= control, Grey= treated*

5.5.6 Lipolysis in mature adipocytes differentiated with and without FPR2/ALX receptor antagonist and/or agonist

In addition to lipogenesis, lipolysis was also investigated. Triglycerides stored in lipid droplets in mature adipocytes are hydrolysed to produce glycerol and free fatty acids. Triglyceride and fatty acid recycling is important in metabolic regulation and is tightly regulated by enzymes such as phosphoenolpyruvate carboxykinase and lipases; adipose triglyceride lipase (*ATGL)*, hormone sensitive lipase (*HSL)* and monoacylglycerol lipase (*MGL)*.

SGBS cells were seeded and induced to differentiate for 14 days with 10µM WRW4 and/or 10µM AC2-26 and mRNA expression levels were studied. mRNA expression levels of *ATGL*, *HSL* and *MGL* were significantly upregulated in mature adipocytes; 5.45 ±1.8-fold, p=0.0334, n=5, 112.3 ±31.4-fold, p=0.0109, n=6 and 4.54 ±1.2-fold, p=0.0175, n=5 respectively compared to preadipocytes (1, n=6), as shown in Figure 5.20. However, mRNA expressions of these lipolytic enzymes were significantly downregulated in mature adipocytes differentiated with antagonist and/or agonist, as shown in Figure 5.20. mRNA expression of *ATGL* was significantly downregulated in WRW4 differentiated mature adipocytes (0.29 ±0.1-fold, p<0.0001, n=5), in AC2-26 differentiated mature adipocytes (0.38 ±0.1-fold, p=0.0006, n=6) and in WRW4 + AC2-26 differentiated mature adipocytes (0.50 ±0.1-fold, p=0.0009, n=4), compared to vehicle control (1, n=6). mRNA expression of *HSL* was significantly downregulated in WRW4 differentiated mature adipocytes (0.41 ±0.03-fold, p<0.0001, n=5) and in AC2-26 differentiated mature adipocytes (0.63 ±0.1-fold, p=0.0205, n=6) compared to vehicle control (1, n=6). Whereas, the mRNA expression levels of *HSL* were upregulated in WRW4 + AC2-26 differentiated mature adipocytes compared to vehicle control, but the difference was not

statistically significant (1.14 ±0.2-fold, p=0.2451, n=6). Furthermore, mRNA expression of *MGL* was significantly downregulated in WRW4 differentiated adipocytes (0.42 ±0.1-fold, p=0.0004, n=7) and in AC2-26 differentiated mature adipocytes (0.63 ±0.1-fold, p=0.0205, n=6) compared to vehicle control (1, n=7). Furthermore, mRNA expression levels were downregulated in WRW4 + AC2-26 differentiated mature adipocytes compared to vehicle control; however, the difference was not statistically significant (0.96 ±0.2-fold vs 1, p=0.4046, n =4).

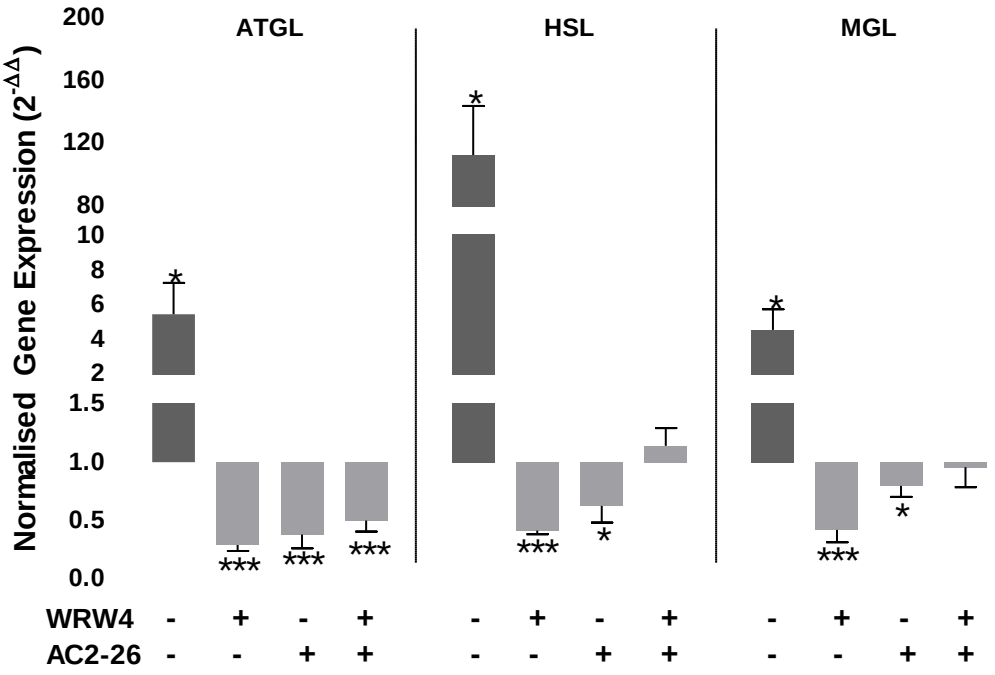


Figure 5.20: mRNA expression of enzymes involved in lipolysis in mature adipocytes differentiated with and without FPR2/ALX antagonist; WRW4 and/or agonist: AC2-26. *SGBS cells were differentiated with 10μM WRW4 and/or 10μM AC2-26 over 14 days to reach a mature adipocyte phenotype. On day 14, mRNA was extracted using Trisure and cDNA was synthesised. 80ng of cDNA was used in SYBR green mediated quantitative PCR. Ct values were obtained and used to calculate normalised gene expression using the Livak $2^{-\Delta\Delta CT}$ equation (as described in Chapter 3). One tailed unpaired student's T-test was used to statistically analyse the data. mRNA expression levels of ATGL, HSL and MGL were significantly upregulated in mature adipocyte; p=0.0334, n=5, p=0.0109, n=6 and p=0.0175, n=5 respectively compared to preadipocytes. However, mRNA expression of these lipolytic enzymes were significantly downregulated in treated mature adipocytes. mRNA expression of ATGL was significantly downregulated in WRW4 differentiated mature adipocytes (p<0.0001, n=5), in AC2-26 differentiated mature adipocytes (p=0.0006, n=6) and in WRW4 + AC2-26 differentiated mature adipocytes (p=0.0009, n=4), compared to DMSO control. mRNA expression of HSL was significantly downregulated in WRW4 differentiated mature adipocytes (p<0.0001, n=5) and in AC2-26 differentiated mature adipocytes (p=0.0205, n=6) compared to vehicle control. Whereas, the mRNA expression levels of HSL were upregulated in WRW4 + AC2-26 differentiated mature adipocytes compared to vehicle control, but the difference was not statistically significant (p=0.2451, n=6). Furthermore, mRNA expression of MGL was significantly downregulated in WRW4 differentiated adipocytes (p=0.0004, n=7) and in AC2-26 differentiated mature adipocytes (p=0.0205, n=6) compared to vehicle control. Furthermore, mRNA expression levels were downregulated in WRW4 + AC2-26 differentiated mature adipocytes compared to vehicle control, however, the difference was not statistically significant (p=0.4046, n=4). Preadipocytes and vehicle control normalised to 1. Data presented as Mean±SEM. P value was set at P<0.05 and is denoted by *vs 1. Dark Grey= control, Grey= treated. Housekeeping gene:18s RNA*
ATGL= adipose triglyceride lipase, HSL= hormone sensitive lipase and MGL= monoacylglycerol lipase

MDA is a by-product of lipid peroxidation and its quantification is used as a marker of lipid peroxidation. In line with the downregulation of mRNA expression of lipolytic enzymes, lipid peroxidation was decreased in mature adipocytes differentiated with WRW4 and/or AC2-26, as shown in Figure 5.21.

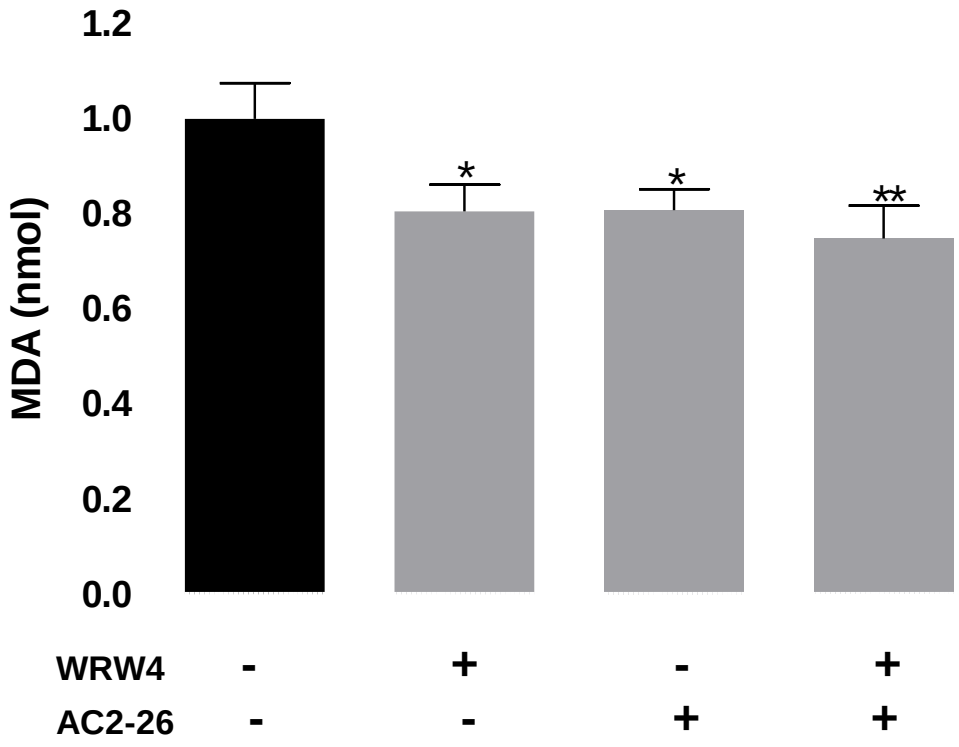


Figure 5.21: Lipid peroxidation analysis in mature adipocytes differentiated with and without FPR2/ALX antagonist; WRW4 and/or agonist: AC2-26. *SGBS cells were differentiated with 10μM WRW4 and/or 10μM AC2-26 over 14 days to reach a mature adipocyte phenotype. On day 14, the cells were lysed using lysis buffer and protein samples were incubated with Thiobarbituric acid for 60 minutes at 95C and the absorbance was measured using a spectrophotometer at 532nm. The data was normalised to control. One tailed unpaired student's T-test was used to statistically analyse the data. Significantly less MDA was present in WRW4 differentiated mature adipocytes (p=0.0450, n=4), in AC2-26 differentiated mature adipocytes (p=0.0085, n=5) and in WRW4 + AC2-26 differentiated mature adipocytes (p=0.0198, n=5), compared to vehicle control. MDA concentration is proportionate to the level lipid peroxidation in mature adipocytes. Data normalised to control and presented as Mean±SEM. P value was set at P<0.05 and is denoted by * vs control. Black= control, Grey= treated.*
MDA= malondialdehyde

Significantly less MDA was present in WRW4 differentiated mature adipocytes (0.84 ±0.1nmol, p=0.0450, n=4), in AC2-26 differentiated mature adipocytes (0.81 ±0.04nmol, p=0.0298, n=5) and in WRW4 + AC2-26 differentiated mature adipocytes (0. 75 ±0.1nmol, p=0.0198, n=5), compared to vehicle control (1 ±0.1nmol, n=5).

5.5.7 Glucose uptake in mature adipocytes differentiated with and without FPR2/ALX receptor antagonist and/or agonist

ANXA1 is thought to regulate insulin secretion and sensitivity, therefore it was speculated whether insulin mediated glucose uptake was affected in mature adipocytes differentiated with WRW4 and/or AC2-26 and whether it was a contributing factor towards decreased lipid accumulation. Therefore, mRNA expression levels of glucose transporters and glucose uptake were assessed.

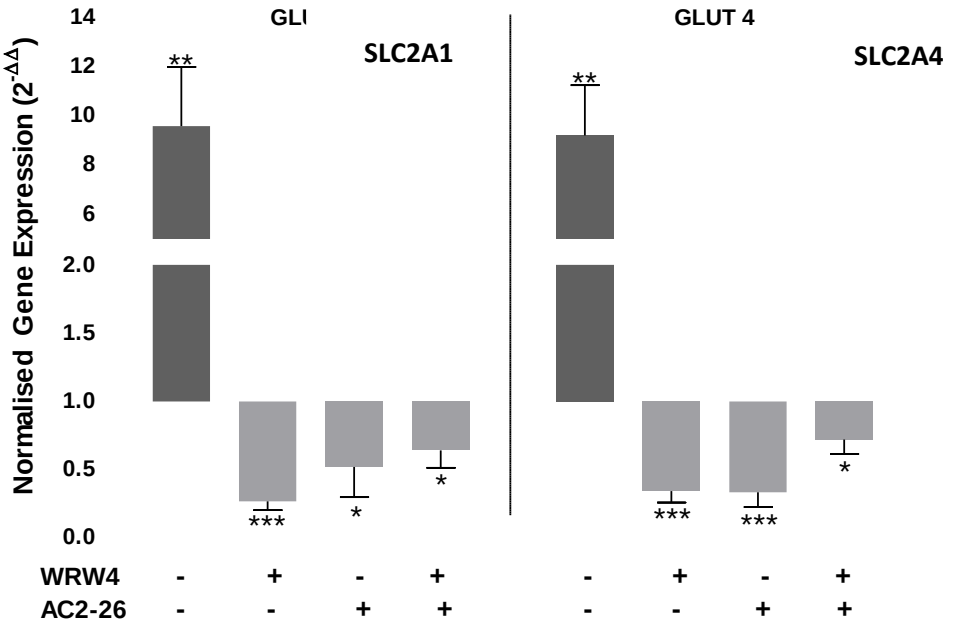


Figure 4.22: mRNA expression of receptors involved in glucose uptake in mature adipocytes differentiated with and without FPR2/ALX antagonist; WRW4 and/or agonist: AC2-26. *SGBS cells were differentiated with 10μM WRW4 and/or 10μM AC2-26 over 14 days to reach a mature adipocyte phenotype. On day 14, mRNA was extracted using Trisure and cDNA was synthesised. 80ng of cDNA was used in SYBR green mediated quantitative PCR. Ct values were obtained and used to calculate normalised gene expression using the Livak $2^{-\Delta\Delta CT}$ equation (as described in Chapter 3). mRNA expression levels of GLUT1 (SLC2A1) and GLUT4 (SLC2A4) were significantly upregulated in mature adipocyte; p=0.0059, n=4 and p=0.0049, n=5 respectively compared to preadipocytes. However, mRNA expression of these receptors was significantly downregulated in treated mature adipocytes. mRNA expression of GLUT1 was significantly downregulated in WRW4 differentiated mature adipocytes (p<0.0001, n=6), in AC2-26 differentiated mature adipocytes (p=0.0316, n=5) and in WRW4 + AC2-26 differentiated mature adipocytes (p=0.0212, n=5), compared to vehicle control. mRNA expression of GLUT4 was significantly downregulated in WRW4 differentiated mature adipocytes (p<0.0001, n=7, in AC2-26 differentiated mature adipocytes (p=0.0001, n=5) and in WRW4 + AC2-26 differentiated mature adipocytes (p=0.267, n=5) compared to vehicle control. Preadipocytes and vehicle control normalised to 1. Data presented as Mean±SEM. * vs 1. Dark Grey= control, Grey= treated, Housekeeping gene:18s RNA*
GLUT= Glucose transporter

SGBS cells were seeded and induced to differentiate for 14 days with 10µM WRW4 and/or 10µM AC2-26 and mRNA expression levels were studied. mRNA expression of glucose transporters; GLUT1 (*SLC2A1)* and GLUT4 (*SLC2A4)* were significantly upregulated in mature adipocytes (9.57 ±2.4-fold, p=0.0059, n=4 and 9.18 ±2.1-fold, p=0.0049, n=5, respectively) compared to preadipocytes (1, n=5), as shown in Figure 5.22. However, mRNA expression levels were significantly downregulated in mature adipocytes differentiated with WRW4 and/or AC2-26, as shown in Figure 5.21. mRNA expression of GLUT1 was significantly downregulated in WRW4 differentiated mature

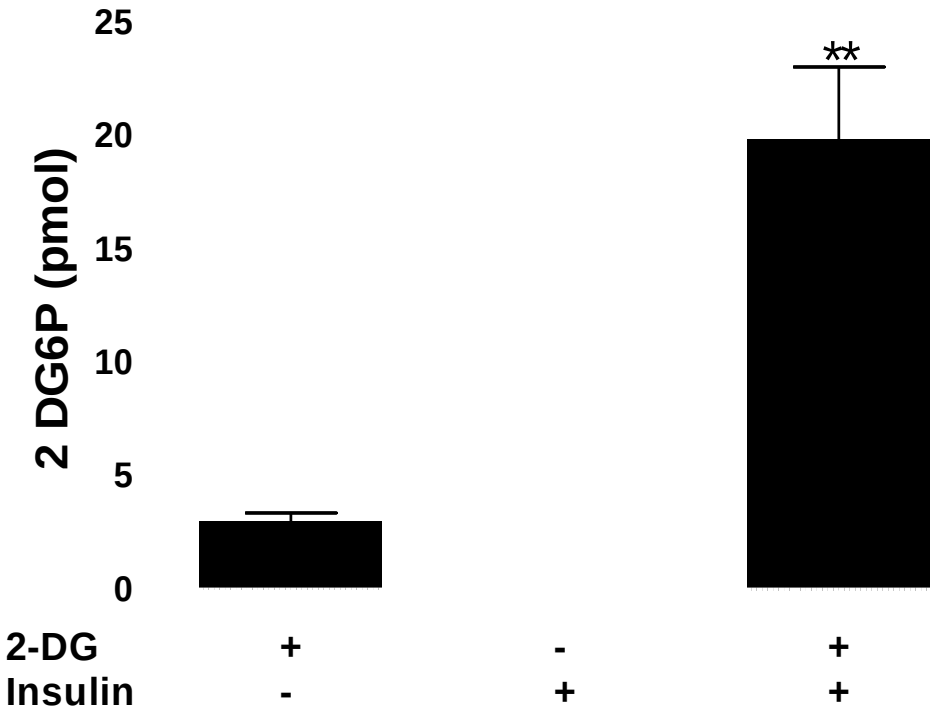


Figure 5.23: Glucose uptake in mature adipose treated with insulin, *SGBS cells were differentiated over 14 days to reach a mature phenotype and maintained for further 4 days. The cells were starved of glucose for 40 minutes and then stimulated with insulin for 20 minutes followed by 2-DG for 20 minutes. The uptake was measured using a spectrophotometer at 412nm. 2-DG is taken up and metabolised to 2DG6P, therefore, the uptake of 2-DG is equivalent to 2-DG6P accumulation. One tailed unpaired student's T-test was used to statistically analyse the data. Glucose uptake significantly increased in adipocytes treated with insulin compared to untreated vehicle control (p=0.0032, n=3). Data presented as Mean±SEM. P value was set at P<0.05 and is denoted by * vs Glucose.*
2-DG= 2-deoxyglucose and 2DG6P= 2-DG-6 phosphate

adipocytes (0.26 ±0.1-fold, p=<0.0001, n=6), in AC2-26 differentiated mature adipocytes (0.52 ±0.2-fold, p=0.0316, n=5), and in WRW4 + AC2-26 differentiated mature adipocytes (0.64 ±0.1-fold, p=0.0212, n=5) compared to vehicle control (1, n=6). Similarly, mRNA expression of GLUT4 were significantly downregulated in WRW4 differentiated mature adipocytes (0.34 ±0.1-fold, p<0.0001, n=7), in AC2-26 differentiated mature adipocytes (0.33 ±0.1-fold, p=0.0001, n=5), and in WRW4 + AC2-26 differentiated mature adipocytes (0.72 ±0.1-fold, p=0.0267, n=5) compared to vehicle control (1, n=6).

Acute treatment of WRW4 and/or AC2-26 decreased glucose uptake in mature adipocytes compared to vehicle control, as shown in Figure 5.27. Glucose uptake significantly increased with insulin treatment in mature adipocytes compared to non-insulin stimulated cells (20.0 ±3.2pmol vs 3.1 ±0.4pmol, p=0.0032, n=3), as shown in Figure 4.23.

Interestingly, 2-DG uptake was significantly increased in non-insulin stimulated adipocytes treated with WRW4 compared to 2DG6P only adipocytes (10.3 ±0.1pmol vs 7.0 ±0.4pmol, p=0.0006, n=3), as shown in Figure 5.24. Furthermore, this was increased in insulin stimulated adipocytes treated with WRW4 compared to non-insulin stimulated WRW4 treated adipocytes (13.8 ±1.4pmol vs 10.3 ±0.1pmol, p=0.0328, n=3) and 2DG6P only adipocytes (13.8 ±1.4pmol vs 7.0 ±0.4, p=0.0045, n=3).

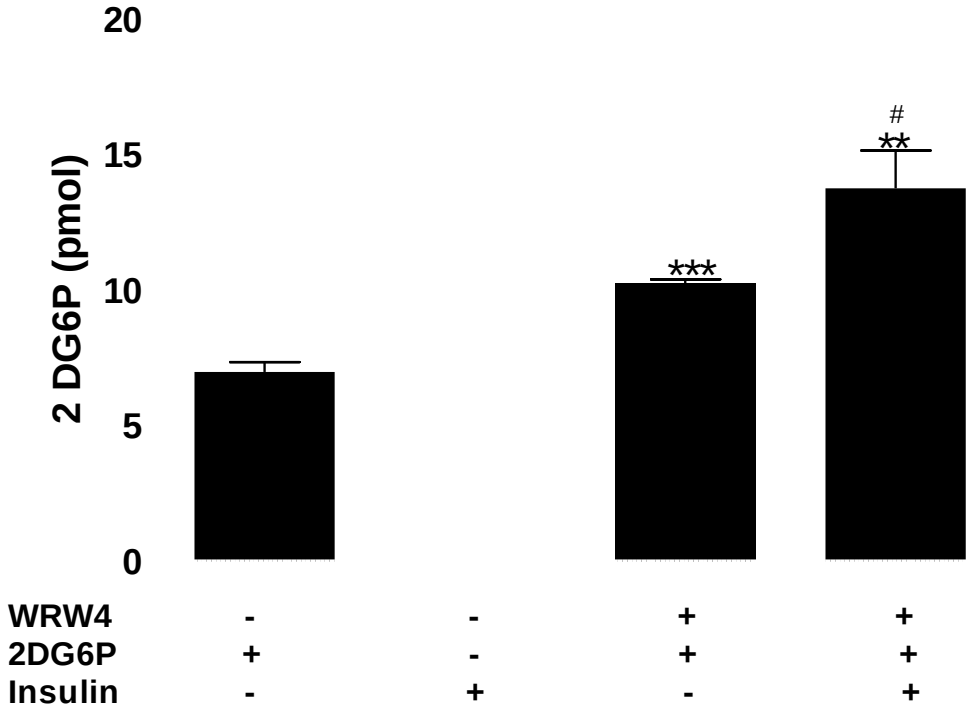


Figure 5.24: Glucose uptake in mature adipocytes treated with insulin and FPR2ALX antagonist; WRW4
*SGBS cells were differentiated over 14 days to reach a mature phenotype and maintained for further 4 days. The cells were starved of glucose for 40 minutes and then stimulated with insulin and 10μM WRW4 for 20 minutes followed by 2-DG for 20 minutes. The uptake was measured using a spectrophotometer at 412nm. 2-DG is taken up and metabolised to 2DG6P, therefore, the uptake of 2-DG is equivalent to 2-DG6P accumulation. One tailed unpaired student's T-test was used to statistically analyse the data. Glucose uptake significantly increased in adipocytes treated with WRW4 compared to 2DG6P only adipocytes (p=0.0006, n=3) and in adipocytes treated with WRW4 and stimulated with insulin compared to unstimulated and WRW4 treated control (p=0.0328, n=3 and p=0.0045, n=3, respectively). Data presented as Mean±SEM. P value was set at P<0.05 and is denoted by * vs 2DG6P only and # vs WRW4 treated.*
2-DG= 2-deoxyglucose and 2DG6P= 2-DG-6 phosphate

A similar pattern of 2-DG uptake was observed in adipocytes treated with AC2-26, as shown in Figure 5.25. Uptake of 2-DG was slightly but not significantly increased in non-insulin stimulated adipocytes treated with AC2-26 compared to 2DG6P only adipocytes (7.8 ±1.1pmol vs 7.0 ±0.4pmol, p=0.2636, n=3). Furthermore, uptake was significantly increased in insulin stimulated AC2-26 treated adipocytes compared to non-insulin

stimulated AC2-26 treated adipocytes (12.1 ±1.1pmol vs 7.8 ±1.1pmol, p=0.0226, n=3) and 2DG6P adipocytes (12.1 ±1.1pmol vs 7.0 ±0.4pmol, p=0.0051, n=3).

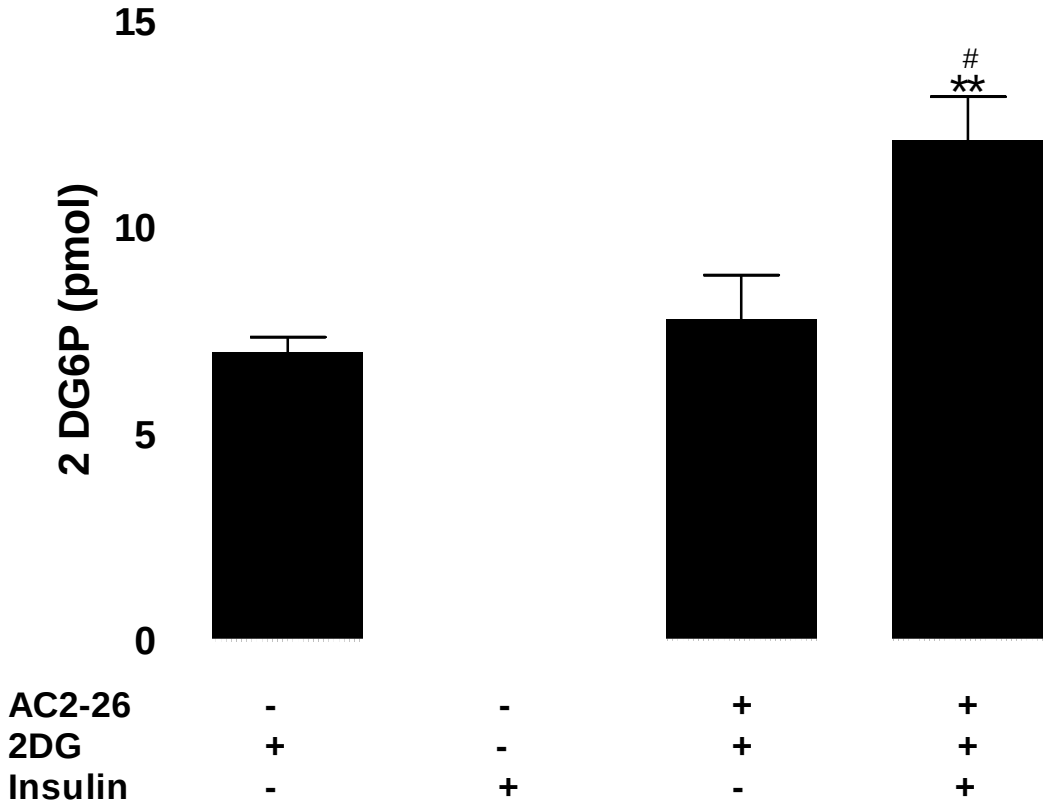


Figure 5.25: Glucose uptake in mature adipocytes treated with insulin and FPR2ALX agonist; AC2-26
*SGBS cells were differentiated over 14 days to reach a mature phenotype and maintained for further 4 days. The cells were starved of glucose for 40 minutes and then stimulated with insulin and 10μM AC2-26 for 20 minutes followed by 2-DG for 20 minutes. The uptake was measured using a spectrophotometer at 412nm. One tailed unpaired student's T-test was used to statistically analyse the data. 2-DG is taken up and metabolised to 2DG6P, therefore, the uptake of 2-DG is equivalent to 2-DG6P accumulation. Glucose uptake significantly increased in adipocytes stimulated with insulin and treated with AC2-26 compared to unstimulated AC2-26 only treated control (p=0.0226, n=3) and 2DG6P glucose only (p=0.0051, n=3). Data presented as Mean±SEM. P value was set at P<0.05 and is denoted by * vs 2GD6P and # vs AC2-26 treated. 2-DG= 2-deoxyglucose and 2DG6P= 2-DG-6 phosphate*

Moreover, a similar pattern of 2-DG uptake was observed in adipocytes treated with WRW4 + AC2-26, as shown in Figure 5.26. Uptake of 2-DG was significantly increased in non-insulin stimulated adipocytes treated with WRW4 + AC2-26 compared to 2GD6P only adipocytes (10.6 ±0.4pmol vs 7.0 ±0.4pmol, p=0.003, n=2/3). Furthermore, this was

increased in insulin stimulated adipocytes treated with WRW4 + AC2-26 compared to non-insulin stimulated WRW4 + AC2-26 adipocytes (16.7 ±1.5pmol vs 10.9 ±0.4pmol, p=0.0293, n=2/3) and 2DG6P only adipocytes (16.7 ±1.5pmol vs 7.0 ±0.4pmol, p=0.0017, n=3).

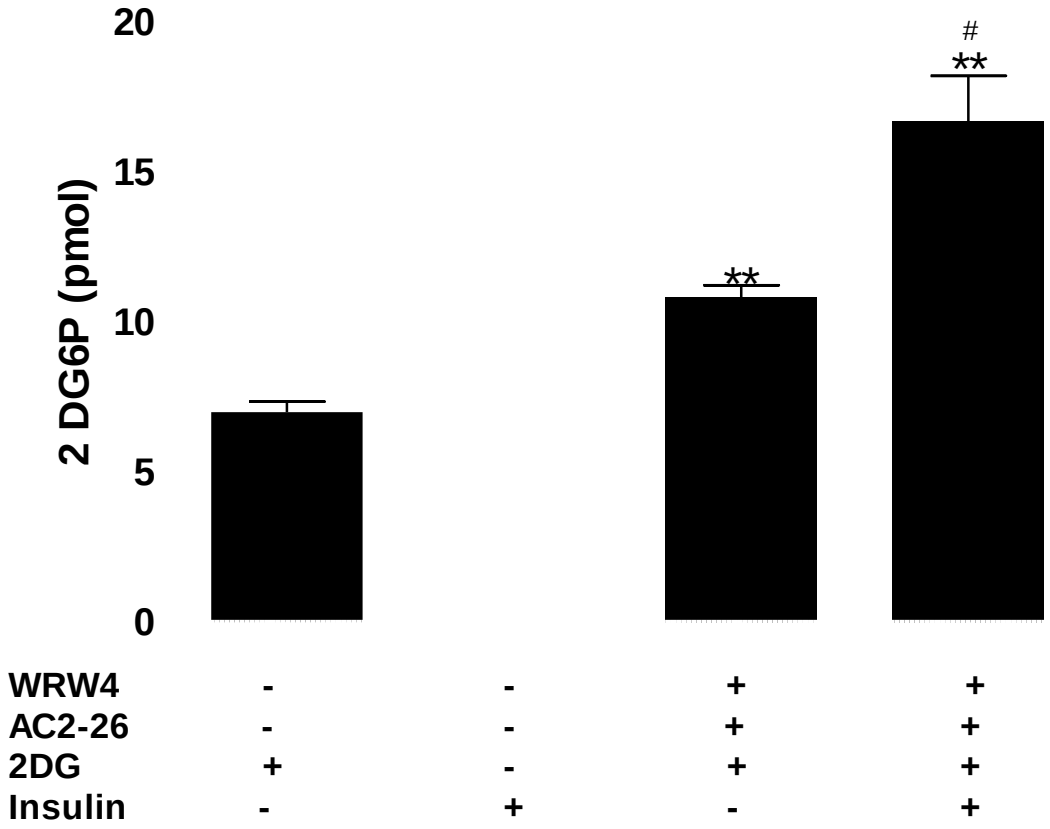


Figure 5.26: Glucose uptake in mature adipocytes treated with insulin and FPR2ALX antagonist; WRW4 and/or agonist; AC2-26 *SGBS cells were differentiated over 14 days to reach a mature phenotype and maintained for further 4 days. The cells were starved of glucose for 40 minutes and then stimulated with insulin, 10μM WRW4 and 10μM AC2-26 for 20 minutes followed by 2-DG for 20 minutes. The uptake was measured using a spectrophotometer at 412nm. One tailed unpaired student's T-test was used to statistically analyse the data. 2-DG is taken up and metabolised to 2DG6P, therefore, the uptake of 2-DG is equivalent to 2-DG6P accumulation. Glucose uptake significantly increased in adipocytes treated with WRW4 + AC2-26 compared to 2DG6P only (p=0.003, n=2/3) and in adipocytes stimulated with insulin and treated with WRW4 + AC2-26 compared to WRW4 + AC2-26 only treated and 2DG6P only adipocytes (p=0.0293, n=2/3 and p=0.0017, n=3, respectively). Data presented as Mean±SEM. P value was set at P<0.05 and is denoted by * vs untreated and # vs AC2-26 treated.*
2-DG= 2-deoxyglucose and 2DG6P= 2-DG-6 phosphate

However, 2-DG uptake was significantly less in insulin stimulated treated adipocytes (WRW; p=0.0222, n=3, AC2-26; p=0.0126, n=3 and WRW4 + AC2-26; p=0.0567, n=3)

compared to insulin stimulated vehicle control adipocytes, as shown in Figure 5.27. Additionally, no significant difference between insulin stimulated treated adipocytes was observed.

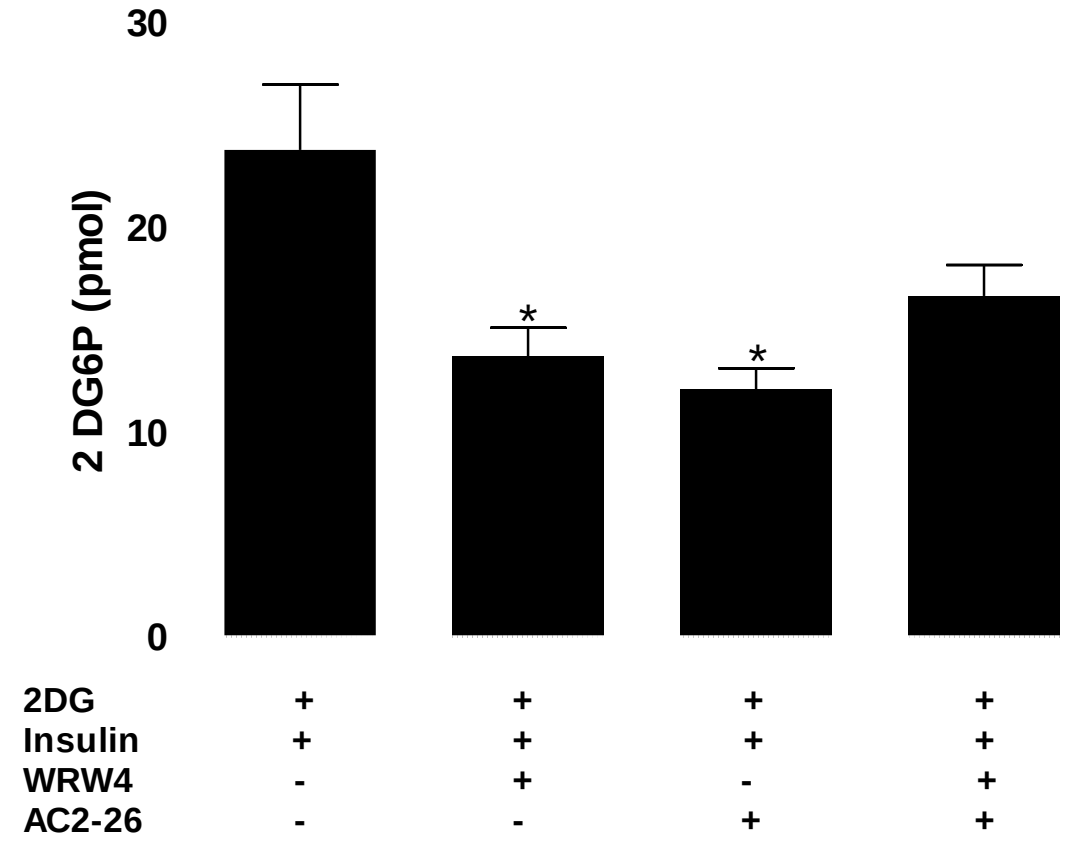


Figure 5.27: Comparison of glucose uptake in adipocytes treated with FPR2/ALX antagonist; WRW4 and/or agonist; AC2-26. *One tailed unpaired student's T-test was used to statistically analyse the data.2-DG was significantly less in treated cells (WRW; p=0.0222, n=3, AC2-26; p=0.0126, n=3 and WRW4 + AC2-26; p=0.0567, n=3) compared to adipocytes treated with insulin only and no significant difference between treated adipocytes was observed. Data presented as Mean±SEM. P value was set at P<0.05 and is denoted by * vs2-DG and insulin only*

5.5.8 mRNA expression of adipokines in mature adipocytes differentiated with and without FPR2/ALX antagonist and/or agonist

Lipid accumulation and expression and secretion of adipokines are positively correlated with adipocyte differentiation. It was questioned whether decreased lipid accumulation was associated with an immature adipocyte phenotype, therefore gene expression of adipokines was investigated. SGBS cells were seeded and induced to differentiate for 14 days with 10µM WRW4 and/or 10µM AC2-26 and mRNA expression levels were studied.

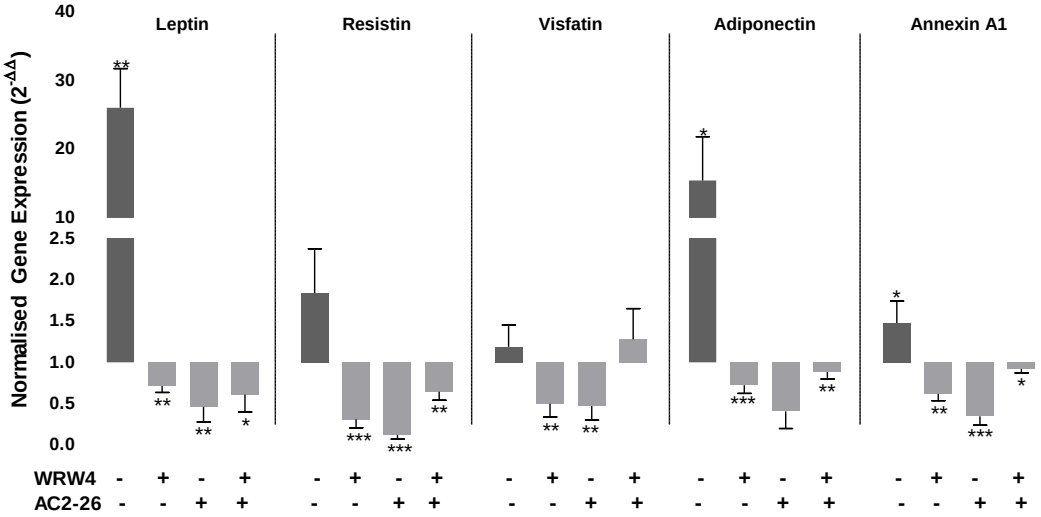


Figure 5.28: mRNA expression of adipokines in mature adipocytes differentiated with FPR2/ALX antagonist; WRW4 and/or agonist; AC2-26. *SGBS cells were differentiated with 10µM WRW4 and/or 10µM AC2-26 over 14 days to reach a mature adipocyte phenotype. On day 14, mRNA was extracted using Trisure and cDNA was synthesised. 80ng of cDNA was used in SYBR green mediated quantitative PCR. Ct values were obtained and used to calculate normalised gene expression using the Livak $2^{-\Delta\Delta CT}$ equation (as described in Chapter 3). One tailed unpaired student's T-test was used to statistically analyse the data. mRNA expression levels of Leptin (p=.0030, n=5), Adiponectin (p=0.0323, n=4) and Annexin A1 (p=0.0438, n=3) were significantly upregulated in mature adipocytes compared to preadipocytes. Similarly, Resistin (p=0.841, n=4) and Visfatin (p=0.2151, n=3) were upregulated in mature adipocytes compared to preadipocytes, however were not statistically significance. mRNA expression levels of these adipokines were significantly downregulated in WRW4 differentiated mature adipocytes; Leptin (p=0.0019, n=4), Resistin (p<0.0001, n=9), Visfatin (p=0.0072, n=5), Adiponectin (p=0.0074, n=4) and Annexin A1 (p=0.0023, n=8) compared to vehicle control. Similarly, mRNA expression levels of these adipokines were significantly downregulated in AC2-26 differentiated mature adipocytes Leptin (p=0.0092, n=5), Resistin (p<0.0001, n=3), Visfatin (p=0.0064, n=5), Adiponectin (p=0.0069, n=4) and Annexin A1 (p=0.0002, n=6) compared to vehicle control. Likewise, mRNA expression levels of these adipokines were significantly downregulated in WRW4 + AC2-26 differentiated mature adipocytes; Leptin (p=0.0379, n=3), Resistin (p=0.0045, n=3) and Annexin A1 (p=0.0484, n=3) compared to vehicle control. mRNA expression levels of Adiponectin (p=0.0770, n=3) were downregulated and Visfatin were upregulated (p=0.2064, n=3) in these cells compared to vehicle control, however, the differences were not statistically significant. Preadipocytes and DMSO control normalised to 1. Data presented as Mean+SEM. P value was set at P<0.05 and is denoted by * vs 1. Dark Grey=*

mRNA expression levels of adipokines were upregulated in mature adipocytes compared to preadipocytes, as shown on Figure 5.28. mRNA expression levels of Leptin (*LEP)*(26.0 ±5.7-fold, p=.0030, n=5), Adiponectin (*ADIPOQ)* (15.4 ±6.4-fold, p=0.0323, n=4) and Annexin A1 (*ANXA1*) (1.5 ±0.3-fold, p=0.0438, n=3) were significantly upregulated in mature adipocytes compared to preadipocytes (1, n=4). Similarly, Resistin (*RETN*) (1.8 ±0.5-fold; p=0.841, n=4) and Visfatin (*NAMPT*) (1.2 ±0.3-fold, p=0.2151, n=3) were upregulated in mature adipocytes compared to preadipocytes, however were not statistically significant.

mRNA expression levels of these adipokines were downregulated in WRW4 differentiated mature adipocytes, as shown in Figure 5.28. mRNA expression levels of Leptin (0.71 ±0.1-fold, p=0.0019, n=4), Resistin (0.25 ±0.1-fold, p<0.0001, n=9), Visfatin (0.50 ±0.1-fold, p=0.0072, n=5), Adiponectin (0.72 ±0.1-fold, p=0.0074, n=4) and Annexin A1 (0.62 ±0.1-fold, p=0.0023, n=8) were significantly downregulated compared to vehicle control (1, n=9).

Similarly, mRNA expression levels of these adipokines were downregulated in AC2-26 differentiated mature adipocytes, as shown in Figure 5.28. mRNA expression levels of Leptin (0.46 ±0.2-fold, p=0.0092, n=5), Resistin (0.12 ±0.1-fold, p<0.0001, n=3), Visfatin (0.50 ±0.2-fold, p=0.0064, n=5), Adiponectin (0.41 ±0.2-fold, p=0.0069, n=4) and Annexin A1 (0.35 ±0.1-fold, p=0.0002, n=6) were significantly downregulated compared to vehicle control (1, n=6).

Likewise, mRNA expression levels of these adipokines were downregulated in WRW4 + AC2-26 differentiated mature adipocytes, as shown in Figure 5.28. mRNA expression levels of Leptin (0.61 ±0.2-fold, p=0.0379, n=3), Resistin (0.64 ±0.1-fold, p=0.0045, n=3)

and Annexin A1 (0.92 ±0.05-fold, p=0.0484, n=3) were significantly downregulated compared to vehicle control (1, n=3). mRNA expression levels of Adiponectin (0.88 ±0.1-fold, p=0.0770, n=3) were downregulated and Visfatin were upregulated (1.28 ±0.4-fold, p=0.2064, n=3) in these cells compared to vehicle control (1, n=3), however, the differences were not statistically significant.

5.6 Discussion

In the current study, the role of ANXA1 in adipocyte differentiation was investigated. The two main *in vivo* studies in murine models published in the area of ANXA1 and adipogenesis reported opposing results; (Warne et al. 2006) conclude ANXA1 may be required for early stages of adipogenesis, namely differentiation from MSC to preadipocytes, whereas (Akasheh et al. 2013) conclude ANXA1 may be involved in regulation of lipolysis in mature adipocytes. In the current study, inhibition of ANXA1 activity did not affect the early stages of adipogenesis in a human primary cell, however, mature adipocytes differentiated with ANXA1 receptor agonist and antagonist accumulated less lipid suggesting, ANXA1 may be involved in modulating the rate of adipogenesis and maintaining a mature adipocyte phenotype. Furthermore, the role of FPR receptors, FPR1 and FPR2/ALX in adipogenesis were investigated as recent studies report. FPR1 agonist fMLF has shown to promote differentiation of hMSC into osteoblasts and supress their differentiation into preadipocytes (Peacey, Wright et al. 2012, Shin, Jang et al. 2011). This observation suggests FPR's expression is upregulated in preadipocytes compared to their precursor cells and continues to upregulate in mature adipocytes as reported by (Kosicka et al. 2013). In this study, differential activation of these receptors resulted in different effect on lipid accumulation in mature adipocytes.

5.6.1 Lipid accumulation

In the current study, differentiating adipocytes with a specific ANXA1 antagonist; WRW4, reduced total lipid accumulation in mature adipocytes, which reflects previous reports describing the reduced fat mass in *ANXA1-/-* mice as reported by (Warne et al. 2006). Interestingly, specifically inhibiting ANXA1 activity or exogenous treatment with

AC2-26 resulted in decreased lipid accumulation in mature adipocytes, but the morphology of the lipid droplets differed. Although, on average, the size of the lipid droplets in mature adipocytes differentiated with specific ANXA1 inhibition was unchanged, morphological analysis revealed increased number of smaller lipid droplets; a phenomenon previously reported in lung fibroblasts, where *ANXA1*$^{-/-}$ mice contained lipid droplets but, *ANXA1*$^{+/+}$ mice did not (Croxtall, Gilroy et al. 2003). Similarly, exogenous treatment with AC2-26 did not result in increased number of smaller lipid droplets. However, on average the size of the lipid droplets was significantly smaller suggesting, ANXA1 may play a role in regulating the size of the lipid droplets and in turn regulating the overall lipid content of mature adipocytes mediating a positive effect as the size and the number of lipid droplets positively correlates with obesity (Engin 2017).

5.6.2 Intracellular Pathways

Interestingly specific inhibition of ANXA1 activity or exogenous treatment with AC2-26 display similar patterns of lipid accumulation in mature adipocytes. Therefore, the roles of ANXA1 receptors in adipogenesis using receptor agonists and antagonists were explored. Previous studies have investigated the role of ANXA1 in adiposity through knockout mice models, however, little evidence is present to suggest ANXA1 or its receptors are involved in adipogenesis. Full length ANXA1 displays specific affinity towards binding and activating the FPR2/ALX receptor, whereas, cleaved peptides such as AC2-26 have shown to bind and activate all members of the FPR family (Dalli, Montero-Melendez et al. 2012). It is interesting to note that, the cleaved peptides of ANXA1 are believed to terminate the anti-inflammatory properties of this protein; binding of ANXA1 to its receptor FPR2/ALX inhibits neutrophil migration, whereas, activation of FPR1 or FPR2/ALX by AC2-26 stimulates neutrophil migration during

inflammation (Dalli, Montero-Melendez et al. 2012, Gavins, Hickey 2012, Hazeldine, Hampson et al. 2015). However, activation of FPR1 and FPR2/ALX have been shown to activate different intracellular signalling cascades, which could be a contributing factor in the differential responses elicited by these receptors.

In the current study, it was hypothesised that blocking the FPR2/ALX receptor may force endogenous AC2-26 to bind to the FPR1 receptor and differentially activate downstream signalling pathways (Figure 5.29a). Adipocytes were differentiated with WRW4 + AC2-26 showed decreased lipid accumulation and an increased number of smaller lipid droplets in mature adipocytes, like that observed in mature adipocytes differentiated with WRW4 only, supporting the hypothesis. Overexpressing AC2-26 may have led to FPR1

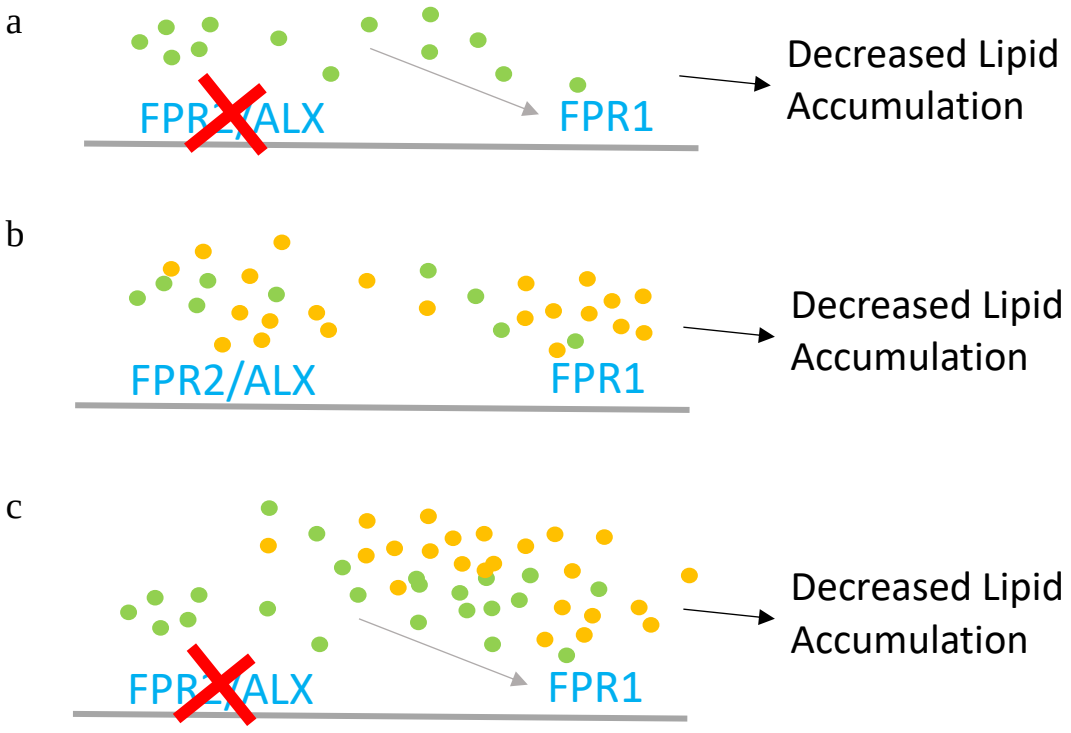


Figure 5.29: Activation of FPR receptors. *a) Specifically blocking FPR2/ALX receptor using WRW4 may force endogenous AC2-26 (green) to bind to FPR1 receptor. b) Activation of both FPR2/ALX and FPR1 receptors using exogenous AC2-26 (yellow) ((Woloszynek, Hu et al. 2012)). c) Specifically blocking FPR2/ALX receptor using WRW4 and overexpression of exogenous AC2-26 to test the hypothesis.*
FPR= Formyl peptide receptor, FPR1= Formyl peptide receptor 1 and FPR2/ALX= Formyl peptide receptor 2

activation along with activation of FPR2/ALX, thus displaying similar effects on lipid accumulation as specifically inhibiting FPR2/ALX receptor alone (Figure 5.29b). Furthermore, blocking FPR1 receptor using lower concentrations of BOC-2 resulted in opposing effects compared to specifically blocking FPR2/ALX receptor suggesting, FPR1 and FPR2/ALX both may act together to regulate and achieve lipid homeostasis in mature adipocytes through heterodimerization or induce crosstalk between intracellular pathways (Kosicka et al. 2013, Dalli et al. 2012). Interestingly, complete inhibition of ANXA1/AC2-26 activity using higher concentrations of BOC-2 showed no difference suggesting, ANXA1 may be involved in regulating adipogenesis in conditions where its dysregulated such as in obesity, (similar to the *ANXA1-/-* mice) not displaying any phenotypic differences unless challenged with an inflammatory stimulus (Akasheh et al. 2013, Warne et al. 2006, Damazo, Yona et al. 2006, Y. H. Yang, Morand et al. 2004, Babbin, Laukoetter et al. 2008).

FPR1 and FPR2/ALX mediate their effects through MAPK signalling. Which specific intracellular MAPK cascades are activated by the individual FPR's is not clear or conclusive further reinforcing the complexity of these signalling pathways. FPR2/ALX activation by ANXA1/AC2-26 is reported to induce ERK1/2 MAPK phosphorylation but not p38 MAPK or JUN MAPK pathways (Bena, Brancaleone et al. 2012), (Dalli et al. 2012, Gavins, Hickey 2012), whereas, others report FPR2/ALX homodimerization by ANXA1/AC2-26 activates the p38 MAPK pathway (Filep 2013) and AC2-26 induced heterodimerisation of FPR1 and FPR2/ALX activates JUN MAPK pathway (Cooray, Gobbetti et al. 2013). Furthermore, ERK1/2 and p38 MAPK predominantly influence chemotaxis of neutrophils, a response exhibited by AC2-26 induced activation of FPR1 (Rabiet, Huet et al. 2007). The contrasting findings of MAPK activation may be cell type

specific and therefore, in this project ERK1/2 MAPK was attributed to FPR2/ALX and p38 MAPK was attributed to FPR1 activation (Figure 5.30) because specific inhibition of FPR2/ALX or exogenous treatment with AC2-26 or both (Figure 5.29) decreased p38 MAPK activity in mature adipocytes. Previously, *ANXA1-/-* mice showed increased p38 MAPK signalling, whereas, treatment with ANXA1 inhibited p38 MAPK expression, further supporting the association of FPR1 and p38 MAPK activity (Y. H. Yang, Toh et al. 2006).

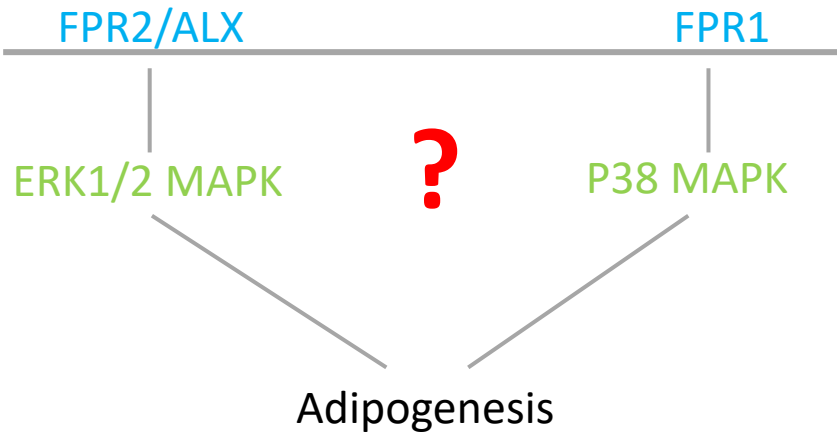


Figure 5.30: Intracellular signalling cascades associated with FPR receptors. *FPR2/ALX is thought to modulate the activity of ERK1/2 MAPK whereas FPR1 is thought to modulate the activity of p38MAPK. ERK1/2 MAPK= FPR1= Formyl peptide receptor 1, FPR2/ALX= Formyl peptide receptor 2, Extracellular signal-regulated kinase mitogen activated protein kinase 1/2, PPARγ Peroxisome proliferator activated receptor γ and C/EBP= CCAAT-enhancer binding proteins*

5.6.2.1 p38 MAPK and adipogenesis

p38 MAPK activity is involved in adipogenesis, however, whether it positively or negatively regulates this process is yet to be decided (Figure 5.31). Phosphorylation of p38 MAPK was decreased in all 3 experimental conditions in the current study, thus reducing its activity.

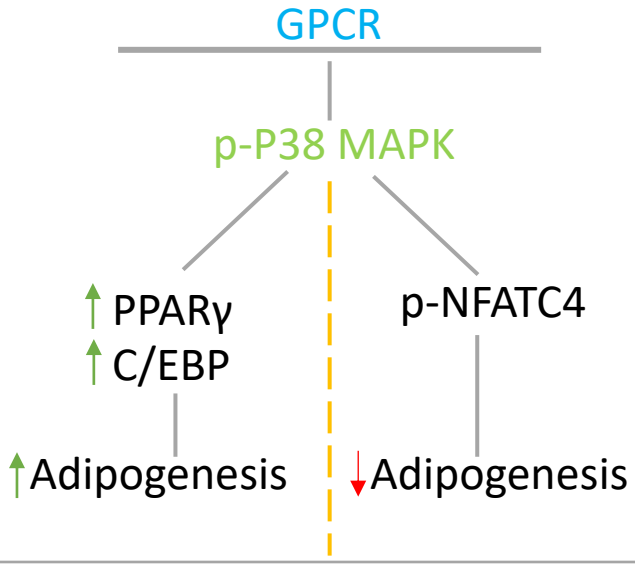


Figure 5.31: Role of p38 MAPK in adipogenesis. *Activated p38 MAPK increases PPARγ and C/EBP activity thus increasing adipogenesis, however, activated p38 MAPK phosphorylates NFATC4; an activator of PPARγ expression in the nucleus. Phosphorylation of NFATC4 inhibits its translocation into the nucleus thus inhibiting adipogenesis.*
GPCR= G-protein coupled receptor, p38 MAPK= p38 mitogen activated protein kinase, PPARγ Peroxisome proliferator activated receptor γ, C/EBP= CCAAT-enhancer binding proteins and NFATC4= Nuclear Factor of activated T cells 4

The first study demonstrating a positive role of p38 MAPK in adipogenesis used 3T3-L1 preadipocytes and reported a decrease in adipocyte formation in the presence of p38 MAPK inhibitors (early on) and associate this effect with decreased C/EBPβ phosphorylation (Engelman, Lisanti et al. 1998). Acute overexpression of mitogen-activated protein kinase 6; an upstream activator of p38 MAPK stimulates adipogenesis, whereas prolonged activation result in adipocyte cell death (Engelman et al. 1998,

Engelman, Berg et al. 1999). Similarly, other authors report decrease in PPARγ activity in the presence of p38 MAPK inhibitors resulting in decreased adipogenesis (Hata, Nishimura et al. 2003, Takenouchi, Takayama et al. 2004), suggesting, p38 MAPK acts as an activator of PPARγ (Hata et al. 2003). However, in human preadipocytes treated with p38 MAPK inhibitors, a smaller but significant decrease in adipogenesis is reported compared to the murine cell line. AC2-26 may act as a p38 MAPK inhibitor as human preadipocytes differentiated with or without p38 MAPK inhibitor, result in decreased lipid accumulation, lipogenesis and leptin secretion in mature adipocytes; similar to the phenotypes observed in the current study (Figure 5.31) (Patel, Holder et al. 2003, Xu, Ju et al. 2014, Aouadi, Laurent et al. 2006, Aouadi, Jager et al. 2007). Furthermore, (Xu et al. 2014) report that not only does p38 MAPK positively regulate gene expression of PPARγ, it also regulates the expression of genes involved in the formation of lipid droplets such as C/EBP, thus decreasing p38 MAPK phosphorylation could be a contributing factor in the decreased number of large lipid droplets observed.

Other authors report a negative role of p38 MAPK in adipogenesis. Phosphorylation of Nuclear Factor of Activated T cells 4, an activator of PPARγ expression in the nucleus, by p38 MAPK inhibits its translocation into the nucleus thus inhibiting adipogenesis (T. T. Yang, Xiong et al. 2002, Ho, Kim et al. 1998). Others report, impaired adipogenesis in 3T3-L1 cells without affecting p38 MAPK activity (S. Liu, Xu et al. 2014). These opposing reports regarding the role of p38 MAPK in adipogenesis suggest, p38 MAPK may have differential roles in the process of adipogenesis depending on the stage of

adipogenesis, differentiation condition and/ or may vary in different cell models (Xu et al. 2014).

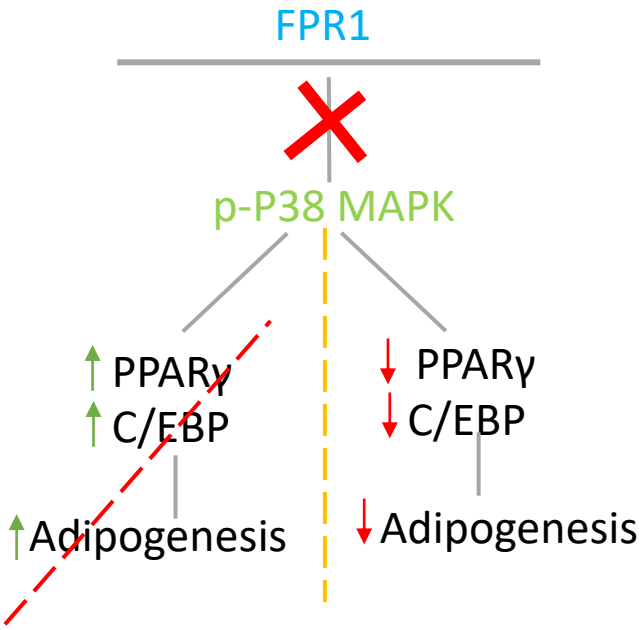


Figure 5.32: Activation of FPR1 decreases p38 MAPK phosphorylation. *Phosphorylated p38 MAPK upregulates the expression of adipogeneic factors such as PPARγ and C/EBP thus positively regulating adipogenesis. However, activation of FPR1 decreases p38 MAPK phosphorylation therefore decrease the expression of PPARγ and C/EBP thus adipogenesis.*
FPR1= Formyl peptide receptor 1, p38 MAPK= p38 kinase mitogen activated protein kinase, PPARγ Peroxisome proliferator activated receptor γ and C/EBP= CCAAT-enhancer binding proteins

Levels of ANXA1 increase in obesity and p38 MAPK activity decreases in both obese human patients and obese animals (Aouadi et al. 2006) (He, Zhu et al. 2013). The results of the current study are the first to link ANXA1 and p38 MAPK activity in adipocyte and may indicate a link between increase in ANXA1 activity, decrease in p38 MAPK activity and dysregulation in adipocyte size in obesity.

5.6.2.2 ERK1/2 MAPK and adipogenesis

Reports investigating the role of ERK1/2 MAPK in adipogenesis also present contradictory results due to ERK1/2 MAPK having multiple roles and each isoform

having distinct biological functions, all contributing to its complex nature (Figure 5.33) (Bost, Aouadi, Caron, and Binetruy 2005). Earlier reports conclude a positive role of ERK1/2 MAPK in adipogenesis (Benito, Porras et al. 1991), whilst later studies demonstrate PPARγ as a substrate of ERK1/2 MAPK and phosphorylation by active ERK1/2 MAPK decreases its transcriptional activity and therefore, inhibits adipocyte differentiation (Camp, Tafuri 1997, Hu, Kim et al. 1996, Ristow, Muller-Wieland et al. 1998). A hypothesis concluded from these results states that function of ERK1/2 MAPK in adipogenesis is regulated in a timely manner; ERK1/2 MAPK is activate for the proliferative step (PPARγ activity not required), whilst, it has to be deactivated in the later stages to avoid phosphorylation of PPARγ (required for terminal differentiation) (Bost et al. 2005). Mice lacking ERK1 MAPK have decreased adiposity and less adipocytes and when challenged with high fat diet (HFD) are resistant to obesity and are protected from insulin resistance (Bost, Aouadi, Caron, Even et al. 2005). Studies in 3T3-L1 and other cell models show that activity of ERK1/2 MAPK is necessary for expression of crucial adipogenic regulators; C/EBPα, β, γ and PPARγ (Prusty, Park et al. 2002, Aubert, Dessolin et al. 1999, Belmonte, Phillips et al. 2001). However, others report upregulation of these adipogeneic factors in 3T3-L1 adipocytes by attenuating ERK1/2 activity thus promoting adipogenesis (C. Liu, Huang et al. 2017).

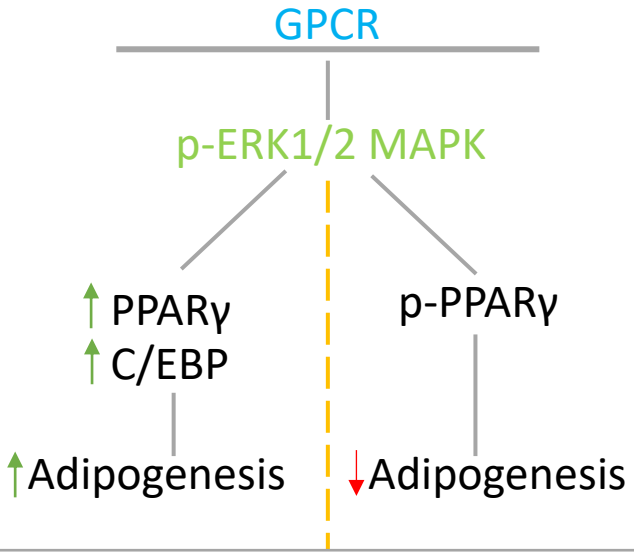


Figure 5.33: Role of ERK1/2 MAPK in adipogenesis. *ERK1/2 MAPK activation induces upregulation of adipogenic factors such as PPARγ and C/EBP thus increasing adipogenesis, however, ERK1/2 MAPK activation phosphorylates PPARγ, inhibiting its translocation into the nucleus thus decreasing adipogenesis.*
GPCR= G-protein coupled receptor, ERK1/2 MAPK= Extracellular signal-regulated kinase mitogen activated protein kinase 1/2, PPARγ Peroxisome proliferator activated receptor γ and C/EBP= CCAAT-enhancer binding proteins

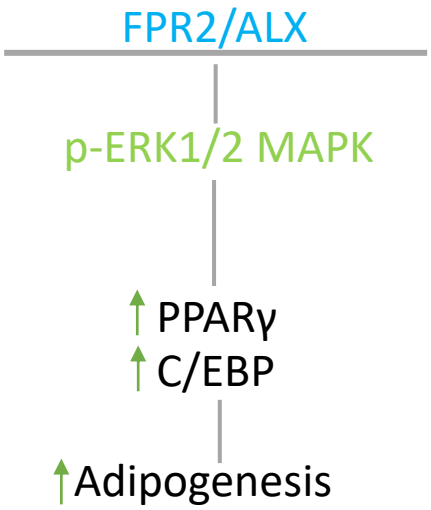


Figure 5.34: Activation of FPR2/ALX may activate ERK1/2 MAPK in mature adipocytes. *Activation of FPR2/ALX by ANXA1/AC2-26 may activate ERK1/2 MAPK and increase expression of lipogenic proteins such as PPARγ and C/EBP therefore, promoting adipogenesis.*
FPR2/ALX= Formyl peptide receptor 2, ANXA= Annexin A1, ERK1/2 MAPK= Extracellular signal-regulated kinase mitogen activated protein kinase 1/2, PPARγ Peroxisome proliferator activated receptor γ and C/EBP= CCAAT-enhancer binding proteins

ERK1/2 MAPK phosphorylation was unaffected in mature adipocytes differentiated with WRW4 and/or AC2-26 compared to control adipocytes. This suggests there may be crosstalk between p38 MAPK and ERK1/2 MAPK, as presented in Figure 5.35b and may explain increased adipogenesis when partially blocking and no difference when completely blocking ANXA1/AC2-26 activity in adipogenesis (Xu et al. 2014). This suggests ERK1/2 MAPK may not be required for adipogenesis in the later stages as reported but may play a positive role if solely activated (Ning, He et al. 2016, Bost et al. 2005). To further validate the hypothesis, the mechanism by which MAPK regulates adipogenesis were investigated.

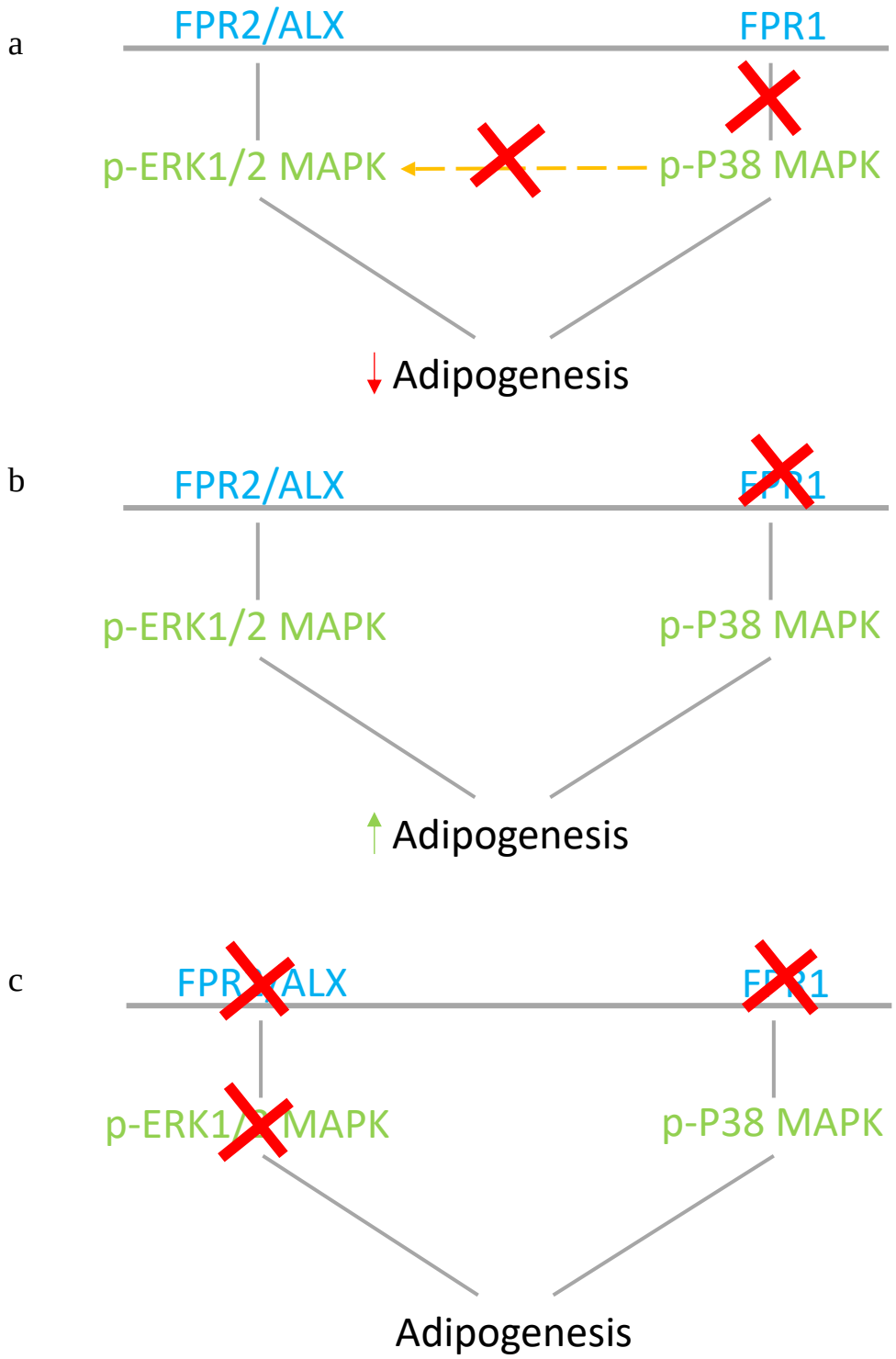


Figure 5.35: Cross talk between p38 MAPK and ERK1/2 MAPK may regulate adipogenesis (hypothesis based on results). *a) Activation of FPR1 decreased p38 MAPK phosphorylation, activation of FPR2/ALX may induce ERK1/2 MAPK activation, however, downregulation of p38 MAPK may block the activation of ERK1/2 MAPK thus decreasing adipogenesis. b) Blocking FPR1 may force endogenous ANXA1/AC2-26 to bind to FPR2/ALX and activate ERK1/2 MAPK thus increasing adipogenesis. c) Blocking FPR1 may increase p38 MAPK activation (adipogenesis) but blocking FPR2/ALX decreases ERK1/2 MAPK phosphorylation (no adipogenesis), therefore, no difference in adipogenesis is observed.*
FPR1= Formyl peptide receptor 1, FPR2/ALX= Formyl peptide receptor 2, ERK1/2 MAPK= Extracellular signal-regulated kinase mitogen activated protein kinase 1/2 and p38 MAPK= p38 mitogen activated protein kinase

5.6.3 Lipid Metabolism in Mature Adipocytes

MAPKs are thought to regulate adipogenesis through peroxisome proliferator activated receptors (PPAR's). PPAR's play important roles in adipogenesis, inflammation, atherogenesis, glucose homeostasis and cancer (Obregon 2014). PPAR's are nuclear receptors that act as transcription factors regulating the expression of proteins involved in lipid metabolism upon stimulation by thiazolidinediones and glitazones (class of insulin-sensitizing drugs). PPARγ is the key regulator of adipose development and adipocyte insulin sensitivity (Kramer, Al-Khalili et al. 2007). Once PPARγ expression is induced, it activates majority of the genes required for this process; aP2 (required for transport of fatty acids), perilipin (covering the surface of the lipid droplet), *de novo* lipid metabolism (including; acetyl-CoA acetyltransferase; ACC, fatty acid synthase; FAS and glycerol-3-phosphate acyltransferase; GPAT) and those involved in glucose homeostasis.

Complete inhibition of ANXA1 signalling did not affect lipid accumulation and previous data report mRNA expression of PPARγ, ACC and FAS in *ANXA1-/-* mice are not differently regulated compared to wild type mice, unless fed a HFD (Akasheh et al. 2013). In accordance to previous reports, decreased phosphorylation of p38 MAPK in mature adipocytes differentiated with WRW4 and/or AC2-26, resulted in decreased mRNA expression of PPARγ and SREBP (Hata et al. 2003) and subsequently, may have downregulated the mRNA expression of enzymes; ACC, FASN and GPAT in the current study. Furthermore, the data in the current study indicated that mature human adipocytes differentiated with WRW4 and/or AC2-26 resulted in decreased total %ACC protein, suggesting that binding of AC2-26 to FPR1 and its activation, downregulates lipogenesis.

Previous *ANXA1-/-* studies conclude ANXA1 may play a role in regulating lipolysis (Warne et al. 2006, Akasheh et al. 2013). In line with this assertion, mature adipocytes differentiated with WRW4 and/or AC2-26 showed decreased mRNA expression of enzymes involved in lipolysis; ATGL, HSL and MGL in the current study. Furthermore, the rate of lipid peroxidation was decreased in mature adipocytes differentiated with WRW4 and/or AC2-26 (Sakamoto, Repasky et al. 1996).

5.6.4 Glucose uptake in Mature Adipocytes

Adipocytes regulate lipid and glucose homeostasis through regulating glucose uptake via adipose specific glucose transporter; GLUT4 and nonspecific glucose transporter; GLUT1. The earliest event in the onset of insulin resistance is the downregulation of GLUT4; an effect which is observed in obesity and T2DM. Clinical studies conducted in overweight diabetic patients report decrease in abundance of ANXA1 protein in the visceral adipose tissue when compared to pre-obese nondiabetic patients (Murri, Insenser et al. 2013). However, it is reported, ANXA1 gene is unlikely to contain variants that contribute to the risk of developing T2DM (Lindgren, Nilsson et al. 2001). Thus, the altered expression may be due to T2DM rather than causing the development of T2DM. It is proposed that ANXA1 could be a potential physiological regulator of insulin action in target tissues as it promotes a dose dependent inhibition of insulin receptor autophosphorylation and stimulation of insulin release in MIN6N8a; an insulin secreting cell line (Lindgren et al. 2001, Won, Kang et al. 2003).

The role of ANXA1 in insulin sensitivity is not clear. A PPARγ agonist, bearing anti-inflammatory and insulin sensitizing properties; Rosiglitazone upregulates ANXA1 expression in the adipose tissue whereas glucose load or exposure to TNFα upregulates

ANXA1 expression in leukocytes, inducing insulin resistance (Ahmed, Neville et al. 2010, Fujimoto, Mochizuki et al. 2010). PPARγ stimulation improves glucose tolerance and insulin sensitivity in T2DM patients in animal models, however, the mechanisms remain unknown (Picard, Auwerx 2002). The downregulation of GLUT4 and GLUT1 mRNA expression in mature adipocytes differentiated with WRW4 or AC2-26 in the current study may be due to downregulation of PPARγ activity in these cells. Although, activation of PPARγ by its agonists upregulate glucose uptake, it does not increase cellular expression of GLUT4, but does increase GLUT1 (Nugent, Prins et al. 2001). Acute treatment with WRW4 and/or AC2-26 increased insulin independent glucose uptake, possibly through GLUT1, however, insulin mediated glucose uptake was reduced compared to control. The data suggests that, ANXA1 may decrease insulin mediated glucose uptake but increase insulin independent glucose uptake in mature adipocytes. However, the data in the current study demonstrates that in addition to GLUT4, GLUT1 mRNA expression was also downregulated in mature adipocytes differentiated with WRW4 and/or AC2-26. Expression of GLUT1 significantly upregulates in obesity and positively correlates with obesity-associated inflammatory cytokines such as IL6 and TNFα (Yin, Gao et al. 2009, Docanto, Ham et al. 2015).

Adipocyte size positively correlates with insulin resistance and the opposite effect has been reported previously. It is well known that inhibition of p38 MAPK improves glucose transport in L6 and 3T3-L1 cells (Furtado, Somwar et al. 2002, Michelle Furtado, Poon et al. 2003), rather than decreased glucose uptake as observed the current study. Furthermore, p38 MAPK activity was previously shown to mediate downregulation of GLUT4 and is higher in isolated adipocytes from T2DM patients (Carlson, Koterski et al. 2003), whereas, in the current study p38 MAPK activity and glucose uptake were

decreased, therefore it is thought that downregulation of GLUT4 may be due to downregulation of PPARγ, as opposed to a direct effect of p38 MAPK.

5.6.5 Adipokines in Mature adipocytes

In addition to its role as a fat storage organ, the adipose tissue serves as an endocrine organ by synthesising and secreting soluble mediators (adipokines) that mediate interaction between the tissue, inflammation and immunity (Tilg, Moschen 2006). Furthermore, adipokines are critical regulators of lipid and glucose homeostasis and mediate crosstalk between the adipose tissue and several other metabolic organs such as, liver, muscle and the pancreas (Cao 2014).

As their synthesis and secretion increases in mature adipocytes compared to preadipocytes, their mRNA expression levels were investigated to determine the maturity of the cells differentiated with WRW4 and/or AC2-26. Leptin regulates feeding behaviours and lipid accumulation, Resistin is implicated in glucose homeostasis and is thought to have a proinflammatory role in humans and similarly, Visfatin plays a role in insulin sensitivity and secretion (Makki, Froguel et al. 2013). All three adipokines positively correlate with adipogenesis and are increased in obesity and T2DM patients (Makki et al. 2013). In the current study, the mRNA expression of Leptin, Resistin and Visfatin were downregulated in mature adipocytes differentiated with WRW4 and/or AC2-26. Furthermore, mRNA expression of Adiponectin; an adipokine deceased in obesity improves insulin resistance and glucose metabolism as it bears insulin sensitizing effects, and ANXA1 were also downregulated in mature adipocytes differentiated with WRW4 and/or AC2-26 (Makki et al. 2013). The decrease in these adipokines may have

affected insulin sensitivity and glucose uptake in these cells. These results indicate an immature adipocyte phenotype compared to control.

5.7 Conclusion

In conclusion, ANXA1 may not be involved in regulating adipogenesis in normal physiological environments, however, when its expression and signalling are dysregulated in conditions such as obesity, it may slow down the processes of adipogenesis to decrease the expression and secretion of proinflammatory adipokines and cytokines associated with a hypoxic environment, thus improving the inflammatory status. However, this effect may be overshadowed by the continued increase in systematic inflammation associated with rapid adipose tissue expansion. In addition to similar experimental limitations as Study 1 (Chapter 4), technical approach used for imaging ORO staining may have produced experimental bias, therefore, it would be of interest to employ an alternative technique or seek technical assistance.

Chapter 6: Plasma inflammatory biomarkers, insulin sensitivity and gut hormones in response to rapid weight loss through Bariatric Surgery

Plasma Annexin A1 inversely correlate with increasing adiposity and with pro-inflammatory cytokines. However, the effect of weight loss on plasma Annexin A1 levels is unknown. Bariatric surgery is associated with reversal of plasma biomarkers including, adipokines, cytokines and gut hormones. Therefore, the objective of this chapter was to investigate the plasma Annexin A1 levels and correlate it with anthropometric measures and plasma inflammatory biomarkers in bariatric surgery patients. Furthermore, the results of Chapter 4 showed AC2-26 upregulated the gene expression of gut hormone; Dipeptidyl Peptidase 4, therefore, plasma gut hormones were correlated with plasma Annexin A1 in the cohort.

6.1 Abstract

Introduction: Bariatric surgery improves metabolic abnormalities independent of weight loss. Changes to the anatomy of the gut induces physiological changes resulting in altered plasma levels of lipids, adipokines, cytokines and gut hormones. The altered circulatory profile leads to improved local and systemic inflammation, however, the exact mechanisms underlying these changes are unclear. Plasma Annexin A1 (ANXA1) levels are reduced in obesity and inversely correlate with markers of adiposity, however, the effect of weight loss plasma ANXA1 levels is unknown.

Aim: To investigate the plasma ANXA1 levels and correlate with plasma lipids, inflammatory biomarkers, markers of insulin sensitivity and gut hormone in response to rapid weight loss induced by bariatric surgery.

Methodology: Patients due to undergo bariatric surgery who matched the inclusion/exclusion criteria were identified and 27 were recruited into the study. Anthropometric measures and blood samples were taken pre-operation (pre-opt), 6 weeks and 3 months post-operation (post-opt) from patients who consented to participate. The participant cohort was further subcategorised based on type of surgery (SG vs RYGB) or type 2 diabetes mellitus (T2DM) status (non-T2DM vs T2DM). Plasma samples were analysed, and results were statistically analysed using GraphPad prism 8.0. Statistical significance at 95% was determined using One-tailed Pearson's correlation coefficient One-way and Two-way ANOVA's followed by Tukey's Post-hoc test.

Results: The average body mass index (BMI) significantly reduced at 6 weeks and 3 months post-opt compared to pre-opt of the complete, SG, RYGB, non-T2DM and T2DM cohorts. Plasma ANXA1 levels increased at 6 weeks and 3 months post-opt compared to pre-opt and correlated with BMI, plasma high density lipoprotein (HDL), tumour necrosis

factor α (TNFα) and adiponectin in the complete cohort. Plasma ANXA1 levels increased at 6 weeks and 3 months post-opt compared to pre-opt in the SG and RYGB cohorts and no significant difference was observed between the cohorts at any time point. In the SG cohort, plasma ANXA1 correlated with BMI, plasma adiponectin and PYY levels. In the RYGB cohort, plasma ANXA1 correlated with plasma triglycerides, HDL, C-reactive protein (CRP), interleukin 1β (IL-1β), TNFα and glucagon-like peptide 1 (GLP-1). Plasma ANXA1 levels increased at 6 weeks and 3 months post-opt compared to pre-opt in the non-T2DM and T2DM cohorts and no significant difference was observed between the cohorts at any time point. In the non-T2DM cohort, plasma ANXA1 correlated with plasma HDL, LDL and homeostatic model assessment of pancreatic β cell function. In the T2DM cohort, plasma ANXA1correlated with BMI, plasma TNFα and homeostatic model assessment of insulin sensitivity.

Conclusion: Plasma ANXA1 indicate increasing trends with decreasing BMI, however, a firm conclusion regarding its associations with plasma biomarkers is difficult to form due to low number of participants.

6.2 Introduction

Bariatric surgery is considered as the most effective long-term treatment option for morbid obese individuals as it has extensive and sustained effects on weight and significantly reduces the risk of developing obesity associated co-morbidities (Colquitt, Picot et al. 2009). It is beneficial for obese individuals with body mass index (BMI) $\geq 40 kg/m^2$ or $\geq 35 kg/m^2$ who have at least 1 co-morbidity present and if non-surgical treatments have failed to achieve or sustain substantial weight loss as outlined by the guidelines published by the National Institute for Health and Clinical Excellence. The primary aim of bariatric surgery is to reduce the patient's total volume of ingestion and/or absorption of contents of food intake, therefore, the procedures are grouped into 3 broad categories, 1) pure gastric restriction, 2) gastric restriction with some malabsorption and 3) gastric restriction with significant intestinal malabsorption (Jaunoo, Southall 2010).

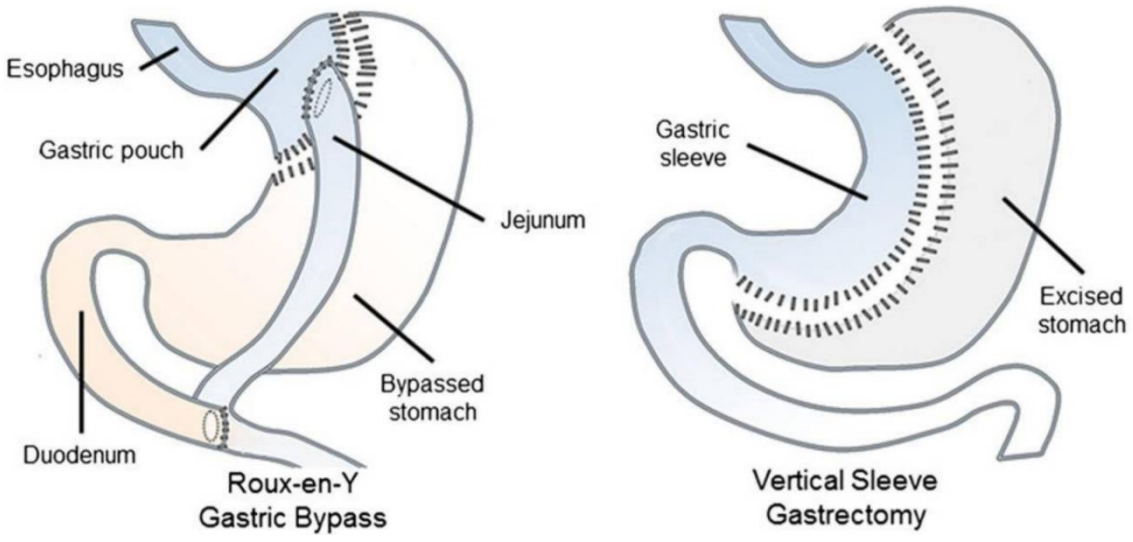


Figure 6.1: Bariatric surgery procedures. *The two common forms of bariatric surgery procedures include, Roux-en-Y Gastric Bypass (RYGB) and Vertical Sleeve Gastrectomy (SG). RYGB combines gastric restriction and malabsorption and involves the division of the stomach into two pouches. The smaller of the pouch is connected to the proximal jejunum bypassing the duodenum. SG is based on gastric restriction and involves the vertical division of the stomach.*

(Frikke-Schmidt, O'Rourke et al. 2016)

The most common procedures include, gastric banding, sleeve gastrectomy (SG), Roux-en-Y gastric bypass (RYGB) and biliopancreatic diversion.

SG is based on gastric restriction and is a less invasive and a less technically challenging procedure compared to RYGB (Frikke-Schmidt, O'Rourke et al. 2016). It is relatively quick to carry out with reduced risk of complications, therefore, was initially offered to high risk bariatric surgery patients (Hayoz, Hermann et al. 2018). It involves vertical division of the stomach reducing its size to about 25% (Figure 6.1). It does not affect gastric function or digestion as it does not involve reconstruction of the pylorus or the intestines. Complications of SG include, leakage from the stomach and vomiting from over-eating. RYGB combines gastric restriction with malabsorption techniques where the stomach is divided into 2 parts; a small proximal pouch (15-20mL) and a large distal pouch. The small proximal pouch is attached to the proximal jejunum, bypassing the large distal gastric pouch and duodenum (Figure 6.1). A small stomach pouch and bypassing the duodenum decreases the digestion and absorption of food, thereby, restricting energy intake (Jaunoo, Southall 2010, Arceo-Olaiz, Espana-Gomez et al. 2008, Yan, Ko et al. 2008). Complications of RYGB include, anastomotic leak, delated gastric emptying, vomiting and dumping syndrome. These procedures were believed to be primarily effective due to their restrictive and malabsorptive properties, however, growing evidence supports the hypotheses that alterations in the gut anatomy have profound effects on the gut physiology, including alterations in gut hormones and their effects on regulating feeding and metabolism (Frikke-Schmidt, O'Rourke et al. 2016). Hormones involved in regulating appetite, satiety and insulin production, such as, gastrointestinal insulinotropic polypeptide (GIP), glucagon-like peptide 1 (GLP-1) and peptide YY (PYY) are

upregulated following surgery, however, the exact mechanism by which bariatric surgery has this effect is unclear (Goktas, Moustaid-Moussa et al. 2013).

Furthermore, bariatric surgery induces significant changes in the anatomical localisation and physiology of the adipose tissue. Up to 50% of the adipose tissue is lost in the first year following bariatric surgery with significant changes in lipid deposition and improvements in metabolic abnormalities (Galanakis, Daskalakis et al. 2015). Hypertrophic adipocytes have altered intracellular functions, induce inflammation and insulin resistance in obesity (Wood, de Heredia et al. 2009). Adipose tissue mass that is lost in response to bariatric surgery is associated with reduced adipocyte hypertrophy, rather than adipocyte hyperplasia, thereby, returning the intracellular functions to baseline and reducing associated abnormalities (Frikke-Schmidt, O'Rourke et al. 2016). Specific changes in adipose tissue depots are also considered to induce metabolic improvements compared to total loss of adipose tissue (Frikke-Schmidt, O'Rourke et al. 2016). For example, visceral adipose tissue (VAT) has increased lipolytic capacity, inflammation, vascularization, increased secretion of adipokines compared to subcutaneous adipose tissue (SAT) and is an independent risk factor for type 2 diabetes mellitus (T2DM) (regardless of fasting insulin levels, insulin secretion, total adiposity and family history) and cardiovascular disease (CVD) (Boyko, Fujimoto et al. 2000, Liu, Fox et al. 2010, Gallagher, Kelley et al. 2009). Reduction in the percentage of VAT induced by bariatric surgery is significantly associated with improvements in insulin sensitivity and remission of T2DM, reduction in flux of free fatty acids and improvements in the inflammatory status (Karki, Farb et al. 2015, Faria, Pestana et al. 2014). These improvements are achieved through multiple pathways associated with systemic and adipose tissue inflammation (Goktas, Moustaid-Moussa et al. 2013).

Adipokines and cytokines are synthesised and secreted by adipocytes and immune cells are active factors that modulate local and systemic metabolism and are detectable in the adipose tissue and the systemic circulation. Dysregulated synthesis and secretion of these adipokines by hypertrophic adipocytes in obesity is hypothesized to contribute to the development of chronic low-grade inflammation, impairing glucose metabolism and insulin sensitivity leading to the onset of insulin resistance and other metabolic co-morbidities (Lindegaard, Jorgensen et al. 2015). In obesity, both circulating and tissue levels of pro-inflammatory proteins are increased, whilst anti-inflammatory proteins are decreased, a situation that is reversed following bariatric surgery, aiding in improving the metabolic profile (Pardina, Ferrer et al. 2012, Viana, Araujo-Dasilio et al. 2013, Sams, Blackledge et al. 2016). Plasma levels and adipocyte expression levels of Annexin A1 (ANXA1) are altered in obesity, however, whether this alteration is reversed with weight loss has not been investigated (Kosicka, Cunliffe et al. 2013). Furthermore, results from Chapter 4 of this thesis suggest, ANXA1 regulates the gene expression of dipeptidyl peptidase 4 (DPP4), thereby possibly modulating the synthesis of gut hormone, GLP-1, therefore, it is of significant interest to investigate the plasma levels of ANXA1 and its effects in response to bariatric surgery.

6.3 Aim, objectives and Hypothesis

The primary aim of this pilot study was to investigate the plasma levels of ANXA1 in response to rapid weight loss through bariatric surgery and correlate it with plasma lipids, plasma inflammatory biomarkers, plasma markers of insulin sensitivity and plasma gut hormone. The objectives of this pilot study were:

- To analyse anthropometric and plasma lipid changes in bariatric surgery patients pre-operatively (pre-opt), 6 weeks and 3 months post-operatively (post-opt)
- To analyse and correlate plasma ANXA1 levels with plasma inflammatory biomarkers, plasma markers of insulin sensitivity and plasma gut hormone in bariatric surgery patients pre-opt, 6 weeks and 3 months post-opt
- To analyse and correlate plasma ANXA1 levels with plasma inflammatory biomarkers, plasma markers of insulin sensitivity and plasma gut hormone by types of bariatric procedures in bariatric surgery patients pre-opt, 6 weeks and 3 months post-opt
- To analyse and correlate plasma ANXA1 levels with plasma inflammatory biomarkers, plasma markers of insulin sensitivity and plasma gut hormone in non-T2DM and T2DM bariatric surgery patients pre-opt, 6 weeks and 3 months post-opt

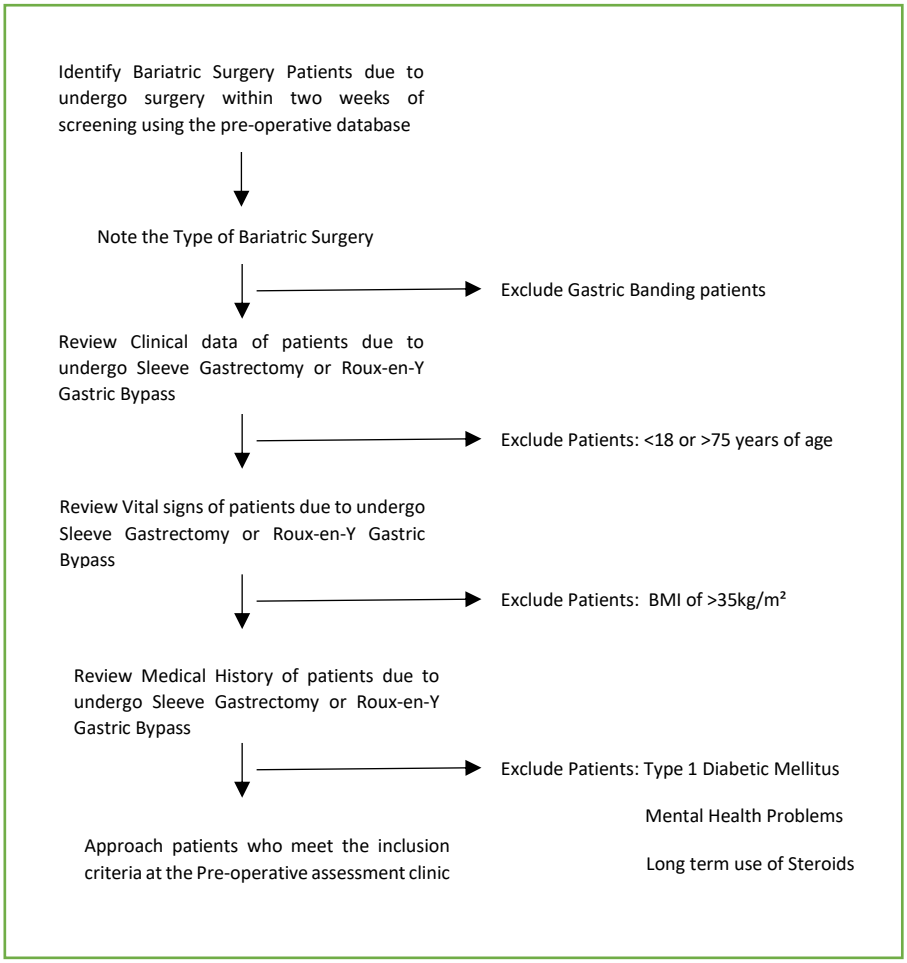
It was hypothesised that weight loss would significantly increase plasma ANXA1 levels, would inversely correlate with pro-inflammatory biomarkers and directly correlate with anti-inflammatory biomarkers, markers of insulin sensitivity and plasma gut hormone, therefore, effectively aiding the resolution of inflammation and decreasing the risk of developing metabolic co-morbidities.

6.4 Materials and Methods

6.4.1 Participants and Experimental design

Following Coventry University (P50481) and NHS (IRAS:220666) ethical approvals, participants were recruited from the general surgery pre-operative assessment clinic at Heartlands Hospital, Birmingham. 52 patients who were scheduled to undergo bariatric surgery (Sleeve Gastrectomy or Roux-en-Y gastric bypass) were screened and identified using the general surgery pre-operative assessment booking database. 34 patients meeting the inclusion criteria (Male/Female adults, 18-75 years old, BMI>40kg/m^2 if do not have T2DM or BMI>35kg/m^2 if known to have T2DM) were approached at their pre-operative assessment appointment and the study design and, the aims were discussed with them (Flow chart 6.1). They were provided with study information sheets and any questions and apprehensions were discussed. 27 patients were happy to take part in the study and provided written consent. Following consent, the baseline clinical assessment (weight and height) was measured by experiences Health Care professionals at the pre-operative assessment clinics and the relevant data (age, gender, past medical history, type of bariatric surgery, co-morbidities, weight, height and BMI) was extracted from patient notes. A number of patients withdrew from the study due to reasons listed below:

Study Recruits		
Total Recruits	**27**	
No of Pre-opt samples	**20/27**	**5 withdrew before pre-opt bloods** - 1 transferred to a different site for opt - 1opted out on their own will - 1 did not meet inclusion criteria (BMI <40kg/m2) + no longer wanted opt. - 2 surgeries cancelled due to other medical reasons **1 withdrew before pro-opt bloods, but decided to continue post-opt** **1; surgery kept rearranging thus missed pre-opt bloods**
No of 6 Weeks Post-opt samples	**16/20**	**4 withdrew before 6 Weeks bloods** - 1 did not attend post opt appoint (did call to arrange but patient was unable to attend) - 1 withdrew after operation - 1 cancelled operation (no longer wanting)
No of 3 Months Post-opt samples	**9/16**	**7 withdrew before 3 Months bloods** - 2 I could not meet - 1 left without seeing me - 2 rearranged - 1 had side effects from OGGT - 1 no show



Flow Chart 6.1: *Flow chart depicting the process of patient recruitment including the inclusion and exclusion criteria.*

Following consent, 10mL of whole blood was collected in purple top anticoagulant-treated tubes (EDTA-treated) via venepuncture for biomarker analysis following their routine bloods by a trained professional (baseline measures). Patients were contacted after their surgery to confirm their willingness to continue their participation. Patients who were happy to continue were met at their 6 weeks post bariatric surgery follow up appointment at the Diabetes and Endocrinology centre at Heartlands Hospital, Birmingham. The clinical assessments (weight, height) and 10mL of whole blood was collected in EDTA-treated tubes via venepuncture for biomarker analysis following routine bloods by a trained professional. Similarly, patients were contacted prior to their 3 months post bariatric surgery follow up to confirm their willingness to continue their participation. Patients who were happy to continue were met at their 3 months post bariatric surgery follow up appointment at the Outpatients Department at Heartlands Hospital, Birmingham. The clinical assessments (weight and height) and 10mL of whole blood was collected in EDTA-treated tubes via venepuncture for biomarker analysis following routine bloods by a trained professional.

Routine blood samples were sent to the local routine pathology lab within the hospital for analysis of the following biochemical measurements; total cholesterol, low density lipoprotein-cholesterol (LDL-C), high density lipoprotein-cholesterol (HDL-C) and triglycerides.

The blood samples collected in EDTA-treated tubes were centrifuged at 1200 rpm for 15 minutes, at 4ºC. The plasma was aliquoted (0.5-1.0mL) into fresh tubes and stored at -80ºC.

6.4.2 Metabolic measurements

6.4.2.1 Enzyme linked immunosorbent Assays

Plasma inflammatory biomarkers, fasting plasma insulin levels and fasting plasma gut hormones were quantified using enzyme linked immunosorbent assay (ELISA). The assays were based on the sandwich technique, as described in Chapter 3 (section 3.3).

- The R&D systems Human C-Reactive Protein (CRP) Quantikine ELISA kit (#DCRP00) was used to quantify CRP in plasma samples, following the manufacturers protocol. The mean intra-assay precision was determined using coefficient of variation (CV). The mean CV of the CRP assay was 2.76%.
- The Cusabio Human Annexin I (ANXAI) ELISA kit (#CSB-E12155h) was used to quantify Annexin A1 levels in plasma samples, following the manufacturers protocol. The mean %CV of the ANXA1 assay was 6.38%.
- The R&D systems Human Interleukin 1β (IL-1β) Quantikine ELISA kit (#DLB50) was used to quantify IL-1β in plasma samples, following the manufacturers protocol. The mean %CV of the IL-1β assay was 4.75%.
- The R&D systems Human Interleukin 10 (IL-10) Quantikine ELISA kit (#D1000B) was used to quantify IL-10 in plasma samples, following the manufacturers protocol. The mean %CV of the IL-10 assay was 6.14%.
- The R&D systems Human Tumour Necrosis Factor α (TNFα) Quantikine ELISA kit (#DTA00D) was used to quantify TNFα in plasma samples, following the manufacturers protocol. The mean %CV of the IL-10 assay was 1.78%.
- The R&D systems Human Leptin Quantikine ELISA kit (#DLP00) was used to quantify Leptin in plasma samples. The mean %CV of the Leptin assay was 3.22%.

- The R&D systems Human Adiponectin Quantikine ELISA kit (#DRP300) was used to quantify Adiponectin in plasma samples. The mean %CV of the Adiponectin assay was 8.89%.
- The DRG systems Human Insulin ELISA kit (#DX-EIA-2935) was used to quantify Insulin in fasted plasma samples. The mean %CV of the insulin assay was 5.05%.
- The Diagenics Human GLP-1 ELISA kit (#EH1053) was used to quantify Insulin in fasted plasma samples, following the manufacturers protocol. The mean %CV of the GLP-1 assay was 9.1%.

Plasma glucose levels were measured using the Biosen C.Line EKF Diagnostic machine following the manufacturers protocol. Briefly, following calibration, 20µL of fasting plasma sample was measured twice. A mean was calculated to determine the concentration of glucose in the patient samples.

6.4.2.2 Calculation of insulin resistance, sensitivity and pancreatic β cell function

The homeostasis model assessment (HOMA) was used to estimate insulin resistance (HOMA-IR), insulin sensitivity (HOMA-%S) and pancreatic β-cell function (HOMA-%β) using fasted glucose and insulin levels. The following equations were used to calculate HOMA-IR, HOMA-%S and HOMA-%β (Glucose is mmol/L and Insulin µIU/mL) (Crofts, C., Wheldon, M., Zinn, C., Lan-Pidhainy, X., Wolever, T., Schofield,G. 2017):

$$\text{HOMA-IR} = (\text{Glucose} \times \text{Insulin}) / 22.5$$

$$\text{HOMA-\%S} = 1/\text{HOMA-\%}\beta$$

$$\text{HOMA-\%}\beta = (20 \times \text{Insulin}) / (\text{Glucose} - 3.5)$$

6.4.3 Statistical analysis

Data presented in this study is expressed as Mean ± Standard Error of the Mean (SEM). GraphPad Prism 8 was used to statistically analyse the data and produce the graphs. One-way and Two-way ANOVA, Tukey's post hoc test and one-tailed Pearson's correlation coefficient were used to statistically analyse the data and to assess the differences in the data sets, a p value of $p<0.05$ was considered statistically significant.

6.5 Results

Anthropometric and plasma samples of consented bariatric surgery patients were analysed. Plasma samples were analysed for plasma lipids, inflammatory biomarkers, markers of insulin sensitivity and gut hormones pre-opt, 6 weeks and 3 months post-operatively post-opt and correlated with plasma ANXA1 levels. The participant cohort was further subdivided into types of bariatric surgery (SG vs RYGB) and T2DM status (non-T2DM vs T2DM) for some of the results presented in this chapter.

6.5.1 Pre and post bariatric surgery: Full cohort

The participant characteristics of the complete cohort are presented in table 6.1. Of the 26 participants at pre-opt, 4 were male and 22 were female, with an average age of 48±1.9. At 6 weeks, 16 females (average age 46±2.6) and at 3 months, 11 females (average age 51±2.7) continued their participation.

Participant Characteristics				
		Pre-Opt	6 Weeks Post-Opt	3 Months Post-Opt
Number of participants		26	16	11
Gender				
	Males	4	0	0
	Females	22	16	11
Age, years				
		48±1.9 (n=26)	46±2.6 (n=16)	51±2.7 (n=11)
Type of Surgery				
	Sleeve Gastrectomy	13	7	5
	Roux-en-Y Gastric Bypass	13	9	6
Type 2 diabetes mellitus				
	Diabetics	10	7	4
	Non-Diabetics	16	9	7
Anthropometric Measures				
	Weight (kg)	131.5±6.4 (n=26)	121.9±5.5 (n=15)	105.9±5.3$^{\#}$ (n=9)
	BMI (kg/m^2)	49.7±1.2 (n=26)	44.6±1.6* (n=15)	38.8±1.5*** (n=9)
Lipid Profiles				
	Triglycerides (mmol/L)	1.3±0.1 (n=25)	1.5±0.1 (n=15)	1.4±0.3 (n=9)
	Total Cholesterol (mmol/L)	4.5±0.1 (n=25)	4.7±0.2 (n=15)	4.5±0.4 (n=9)
	HDL (mmol/L)	1.2±0.1 (n=25)	1.1±0.1 (n=15)	1.4±0.1$^{\#}$ (n=8)
	LDL (mmol/L)	2.7±0.1 (n=25)	2.9±0.2 (n=15)	2.5±0.2 (n=9)

Table 6.1: Participant characteristics at pre and post- Bariatric surgery. *The anthropometric measures and lipid profiles were analysed in participants undergoing bariatric surgery at pre-, 6 weeks and 3 months post-operatively. One-way ANOVA followed by Tukey's Post-hoc multiple comparison test was used to statistically analyse the data. Data presented as Mean±SEM. P value was set at p <0.05 and is denoted by *vs pre opt, #vs 6 weeks*
Pre-opt; Pre-operatively, Post-opt; Post-operatively, BMI; Body mass index, HDL; High density lipoprotein and LDL; Low density lipoprotein

6.5.1.1 Anthropometric and plasma lipid levels

On average, the average weight was significantly reduced at 3 months post-opt compared to pre-opt (131.5±6.4 kg, n=26 vs 105.9±5.3 kg, n=9, p=0.049, respectively) (Table 6.1). Similarly, the average BMI was significantly reduced at 6 weeks and 3 months post-opt compared to pre-opt (44.6±1.6 kg/m^2, n=15, p=0.025 and 38.8±1.5 kg/m^2, n=9, p<0.0001 vs 49.7±1.2 kg/m^2, n=26, respectively), as shown in Figure 6.2.

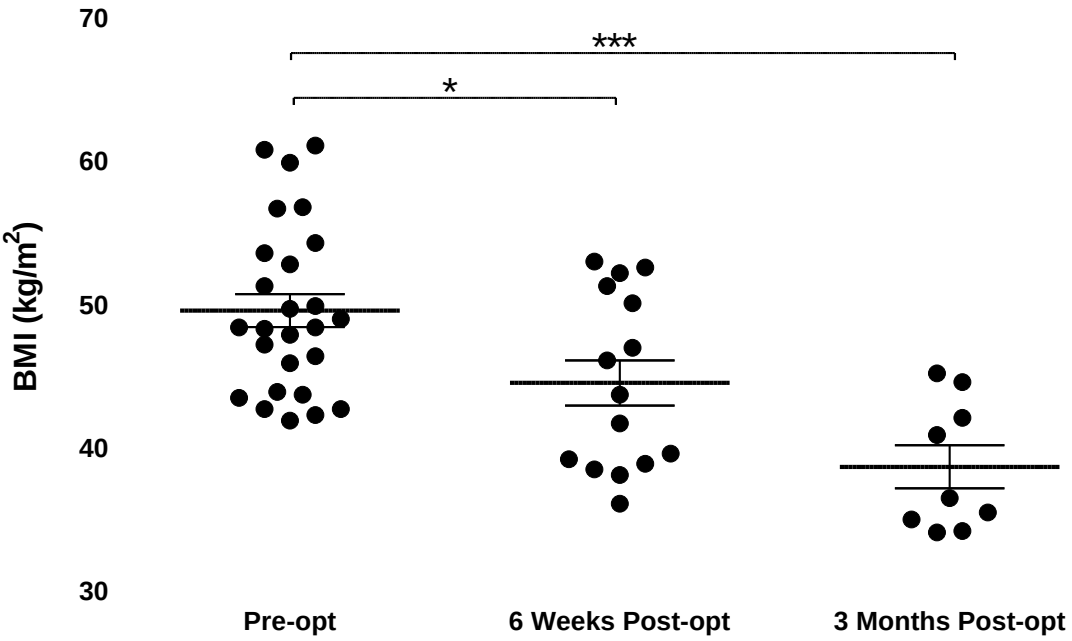


Figure 6.2: Average BMI (kg/m^2) of participant cohort pre- and post- Bariatric surgery. *The average Body mass index was significantly reduced at 6 weeks and 3 months post-opt compared to pre-opt (p=0.025, n=15 and p<0.0001, n=9, respectively). One-way ANOVA followed by Tukey's Post-hoc multiple comparison test was used to statistically analyse the data. Data presented as Mean±SEM. P value was set at p <0.05. BMI= Body mass index, Pre-opt= Pre-operatively and Post-opt= post-operatively*

The changes in the average plasma lipid levels of triglycerides, total cholesterol and LDL post-opt compared to pre-opt did not significantly differ (Table 6.1). However, the average plasma HDL levels significantly improved at 3 months post-opt compared to 6

weeks post-opt (1.4±0.1 mmol/L, n=8 vs 1.1±0.1 mmol/L, n=15, p=0.044, respectively) (Table 6.1).

6.5.1.2 Plasma inflammatory biomarkers, markers of insulin sensitivity and gut hormone

Table 6.2 presents the analysis of plasma inflammatory biomarkers, plasma markers of insulin sensitivity and plasma gut hormones pre- and post- bariatric surgery of the complete participant cohort.

6.5.1.2.1 Plasma inflammatory Biomarkers

On average, plasma C-reactive protein (CRP) levels decreased at 6 weeks and 3 months post-opt compared to pre-opt, however, the difference was not statistically significant (10.1±2.3 µg/mL, n=14 and 8.0±3.7 µg/mL, n=6 vs 11.6±2.4 µg/mL, n=18, respectively). On average, the plasma interleukin-1β (IL-1β) levels significantly increased at 3 months post-opt compared to pre-opt (2.2±0.3 pg/mL, n=9 vs 1.3±0.2 pg/mL, n=17, p=0.019, respectively) and 3 months post-opt compared to 6 weeks post-opt (2.2±0.3 pg/mL, n=9 vs 1.3±0.2 pg/mL, n=14, p=0.027, respectively). On average, the plasma interleukin-10 (IL-10) levels significantly increased at 6 weeks post-opt compared to pre-opt (9.3±2.2 pg/mL, n=13 vs 6.9±0.7 pg/mL, n=18, p=0.048, respectively) and remained at a similar level at 3 months post-opt compared to 6 weeks post-opt (9.1±0.8 pg/mL, n=8 vs 9.3±0.6 pg/mL, n=13, respectively). On average, the plasma tumour necrosis factor α (TNFα) levels remained similar at 6 weeks and 3 months post-opt compared to pre-opt (21.2±0.8 pg/mL, n=15 and 21.7±1.4 pg/mL, n=9 vs 21.7±0.4 pg/mL, n=19, respectively). On average, the plasma leptin levels significantly decreased at 6 weeks and 3 months post-

opt compared to pre-opt (34.6±5.0 ng/mL, n=15, p<0.0001 and 35.7±4.8 ng/mL, n=9, p=0.0001 vs 80.9±6.8 ng/mL, n=20, respectively). On average, the plasma adiponectin

Plasma Biomarkers: Full Cohort				
		Pre-Opt	6 Weeks Post-Opt	3 Months Post-Opt
Inflammatory Biomarkers				
	CRP (µg/mL)	11.6±2.4 (n=18)	10.1±2.3 (n=14)	8.0.6±3.7 (n=6)
	Annexin A1 (ng/mL)	1.1±0.1 (n=20)	1.4±0.2 (n=16)	1.6±0.1 (n=9)
	IL-1β (pg/mL)	1.3±0.2 (n=17)	1.3±0.2 (n=14)	2.2±0.3*$^{\#}$ (n=9)
	IL-10 (pg/mL)	6.9±0.7 (n=18)	9.3±2.2* (n=13)	9.1±0.8 (n=8)
	TNFα (pg/mL)	21.7±0.4 (n=19)	21.2±0.8 (n=15)	21.7±1.4 (n=9)
	Leptin (ng/mL)	80.9±6.8 (n==20)	34.6±5.0*** (n=15)	35.7±4.8*** (n=9)
	Adiponectin (µg/mL)	5.3±0.6 (n=19)	8.1±0.8* (n=13)	8.9±0.8** (n=8)
Insulin Sensitivity				
	Fasting Glucose (mmol/L)	4.2±0.3 (n=18)	3.9±0.1 (n=9)	4.2±0.8 (n=7)
	Fasting Insulin (µIU/mL)	25.7±1.9 (n=19)	18.1±1.9* (n=9)	14.7±2.4** (n=7)
	HOMA-IR	3.2±0.3 (n=15)	2.2±0.2 (n=9)	1.8±0.3 (n=7)
	HOMA-%S (%)	34.0±2.5 (n=15)	44.6±3.1 (n=8)	57.5±2.47.6*** (n=6)
	HOMA-%β (%)	321.0±27.0 (n=14)	260.4±10.6 (n=9)	252.2±24.0 (n=6)
Gut Hormone				
	Fasting GLP-1 (ng/mL)	264.2±12.0 (n=19)	311.3±16.1 (n=9)	354.5±17.6*** (n=7)

Table 6.2: Plasma biomarkers analysis of participant cohort at pre- and post- Bariatric surgery. *Plasma inflammatory biomarkers, fasting plasma glucose and insulin levels and plasma gut hormone were analysed and, HOMA-IR, HOMA-%S and HOMA-%β were calculated using the equations presented in section 6.4.2.2. One-way ANOVA followed by Tukey's Post-hoc multiple comparison test was used to statistically analyse the data. Data presented as Mean±SEM. P value was set at p <0.05 and is denoted by *vs pre opt, $^{\#}$vs 6 weeks of the corresponding group*
Pre-opt= Pre-operatively, Post-opt= post-operatively, CRP= C-reactive protein, IL-= Interleukin, TNFα= Tumour necrosis factor α, HOMA-IR= Homeostatic model assessment of insulin resistance, HOMA-%S= Homeostatic model assessment of insulin sensitivity, HOMA-%β= Homeostatic model assessment of pancreatic β cell function and GLP-1= Glucagon like peptide 1

levels significantly increased at 6 weeks and 3 months post-opt compared to pre-opt (8.1±0.8 µg/mL, n=13, p=0.018 and 8.9±0.8 µg/mL, n=8, p=0.009 vs 5.3±0.6 µg/mL, n=19, respectively).

6.4.1.2.2 Plasma markers of insulin sensitivity

On average, the fasting plasma glucose levels remained similar at 6 weeks and 3 months post-opt compared to pre-opt (3.9±0.1 mmol/L, n=9 and 4.2±0.8 mmol/L, n=7 vs 4.2±0.3 mmol/L, n=18, respectively). However, the average fasting plasma insulin levels significantly decreased at 6 weeks and 3 months post-opt compared to pre-opt (18.1±1.9 µIU/mL, n=9, p=0.047 and 14.7±2.4 µIU/mL, n=7, p=0.006 vs 25.7±1.9 µIU/mL, n=19, respectively). On average, HOMA-IR significantly reduced at 6 weeks and 3 months post-opt compared to pre-opt (2.2±0.2, n=9, p=0.021 and 1.8±0.3, n=7, p=0.003 vs 3.2±0.3, n=15, respectively). On average, HOMA-%S significantly improved at 3 months post-opt compared to pre-opt (57.5±7.6 %, n=6 vs 34±2.5 %, n=15, p=0.0009, respectively). On average, HOMA-%β reduced at 6 weeks and 3 months post-opt compared to pre-opt, however, the difference was not statistically significant (260.4±10.6 %, n=9 and 252.2±24.0 %, n=6 vs 321±27.0 %, n=14, respectively).

6.5.1.2.3 Plasma gut hormone

On average, the fasting plasma GLP-1 levels significantly increased at 3 months post-opt compared to pre-opt (354.5±17.6 ng/mL, n=7 vs 264.2±12.0 ng/mL, n=19, p=0.0003 respectively).

6.5.1.3 Annexin A1

On average, the plasma ANXA1 levels increased at 6 weeks and 3 months post-opt compared to pre-opt, however, the difference was not statistically significant in the full cohort (1.4±0.2 ng/mL, n=16, p=0.080 and 1.6±0.1 ng/mL, n=9, p=0.170, vs 1.1±0.1 ng/mL, n=20, respectively), as shown in Figure 6.3.

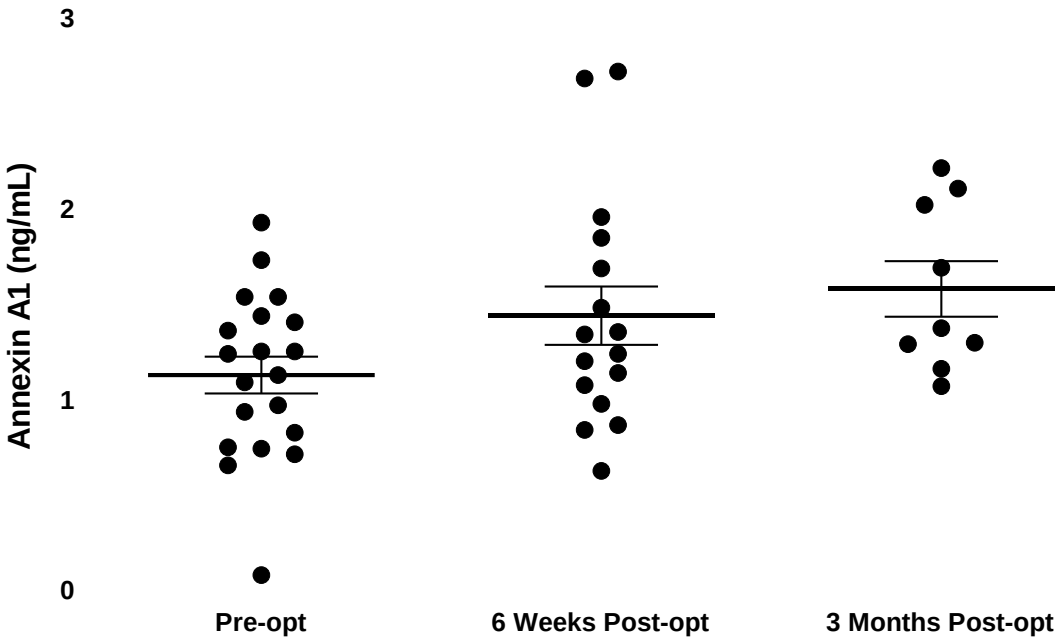


Figure 6.3: Average plasma Annexin A1 (ng/mL) levels of participant cohort pre- and post- Bariatric surgery. *The average plasma Annexin A1 levels increased at 6 weeks and 3 months post-opt compared to pre-opt (p=0.080, n=16 and p=0.170, n=9, respectively). One-way ANOVA followed by Tukey's Post-hoc multiple comparison test was used to statistically analyse the data. Data presented as Mean±SEM. P value was set at p <0.05.*

Plasma ANXA1 levels inversely correlated with BMI (r=0.219, p=0.077, n=44) (Figure 6.4), directly correlated with plasma HDL levels (r=0.242, p=0.064, n=41) (Figure 6.5), significantly directly correlated with plasma TNFα levels (r=0.321, p=0.018, n=43) (Figure 6.6) and directly correlated with plasma adiponectin levels (r=0.226, p=0.083, n=39) (Figure 6.7), in the complete cohort.

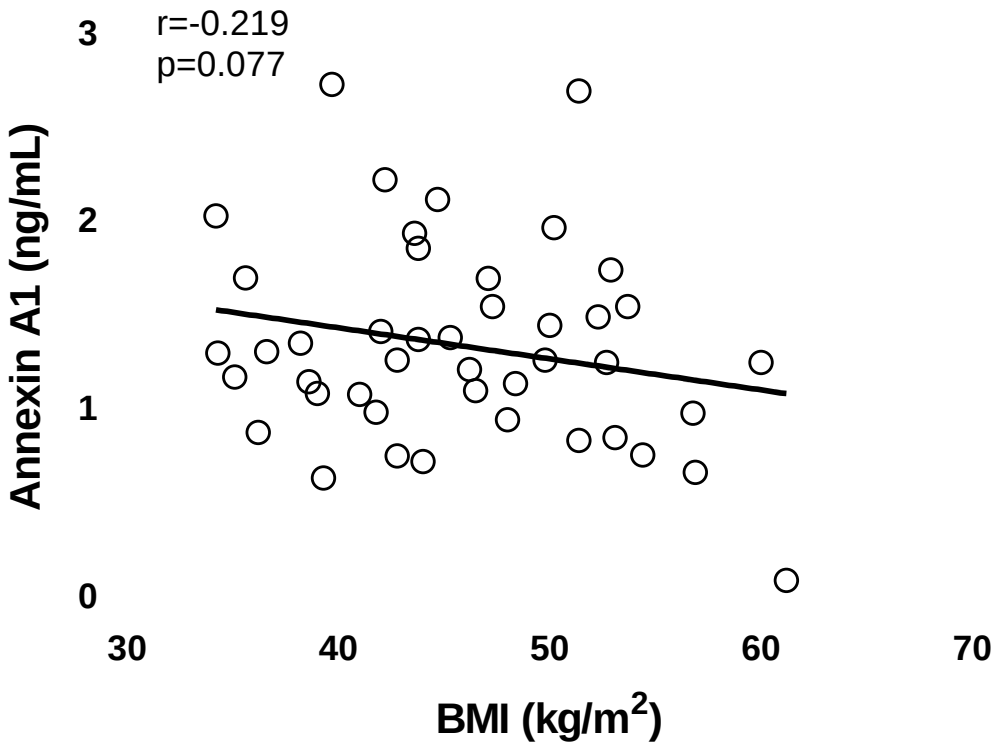


Figure 6.4: Correlation between plasma Annexin A1 levels and BMI in participant cohort. *Plasma Annexin A1 levels inversely correlated with BMI. One tailed Person correlation coefficient was used to statistically analyse the data.*
BMI= Body mass index

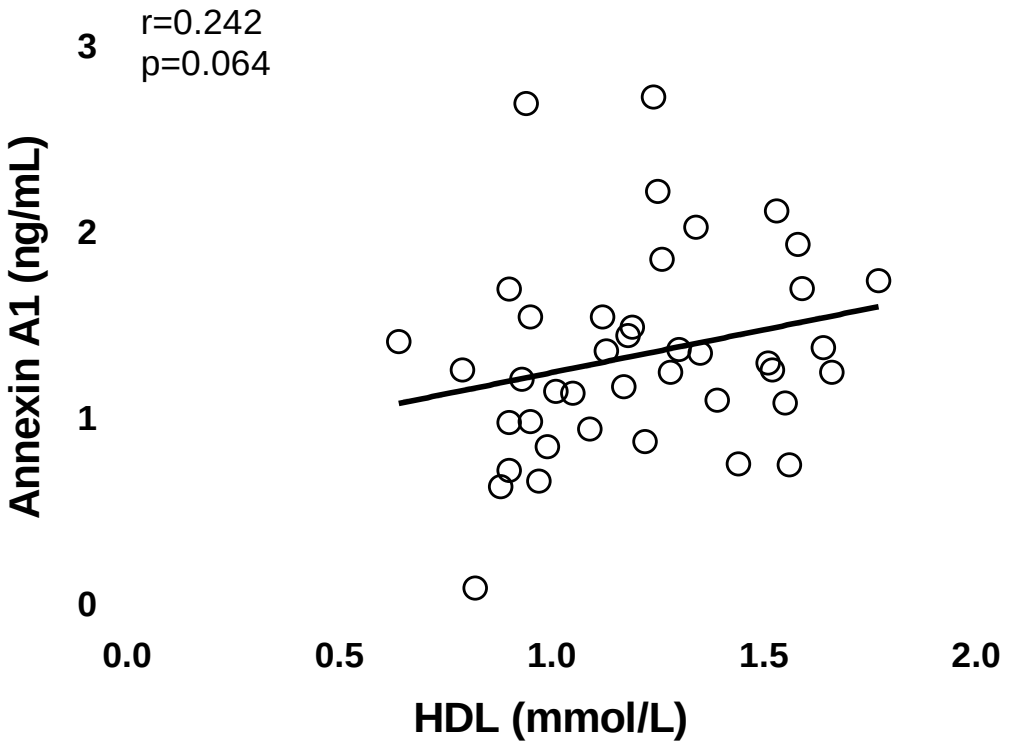


Figure 6.5: Correlation between plasma Annexin A1 levels and plasma HDL levels in participant cohort.
Plasma Annexin A1 levels directly correlated with plasma HDL levels. One tailed Person correlation coefficient was used to statistically analyse the data.
HDL= High density lipoprotein

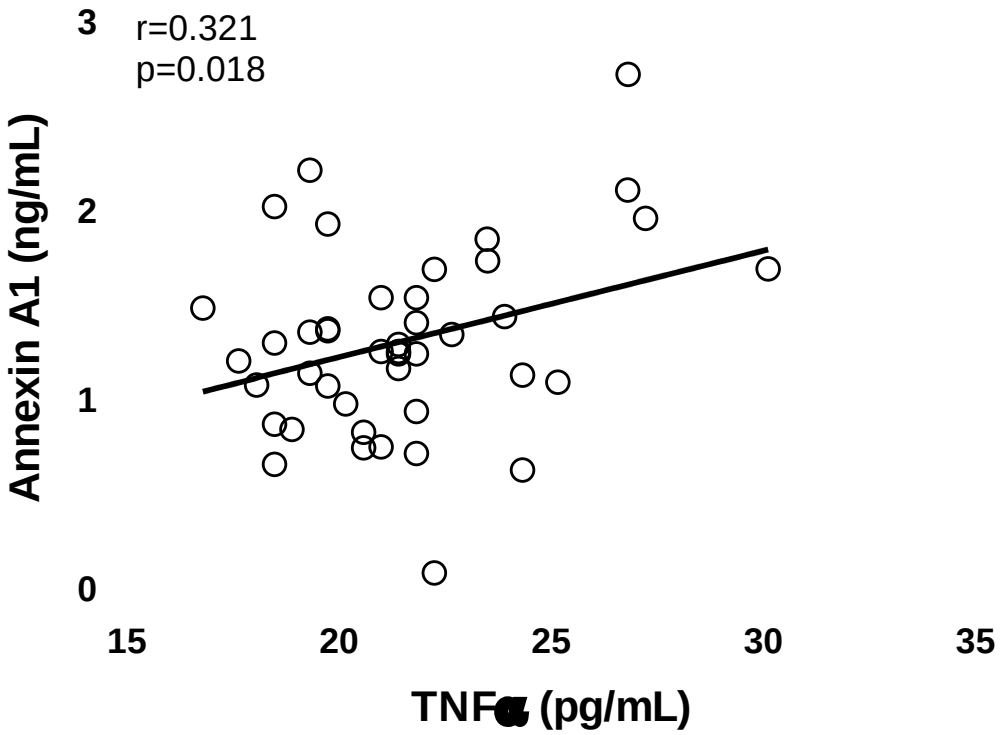


Figure 6.6: Correlation between plasma Annexin A1 levels and plasma TNFα levels in participant cohort.
Plasma Annexin A1 levels significantly directly correlated with plasma TNFα levels. One tailed Person correlation coefficient was used to statistically analyse the data.
TNFα= Tumour necrosis factor α

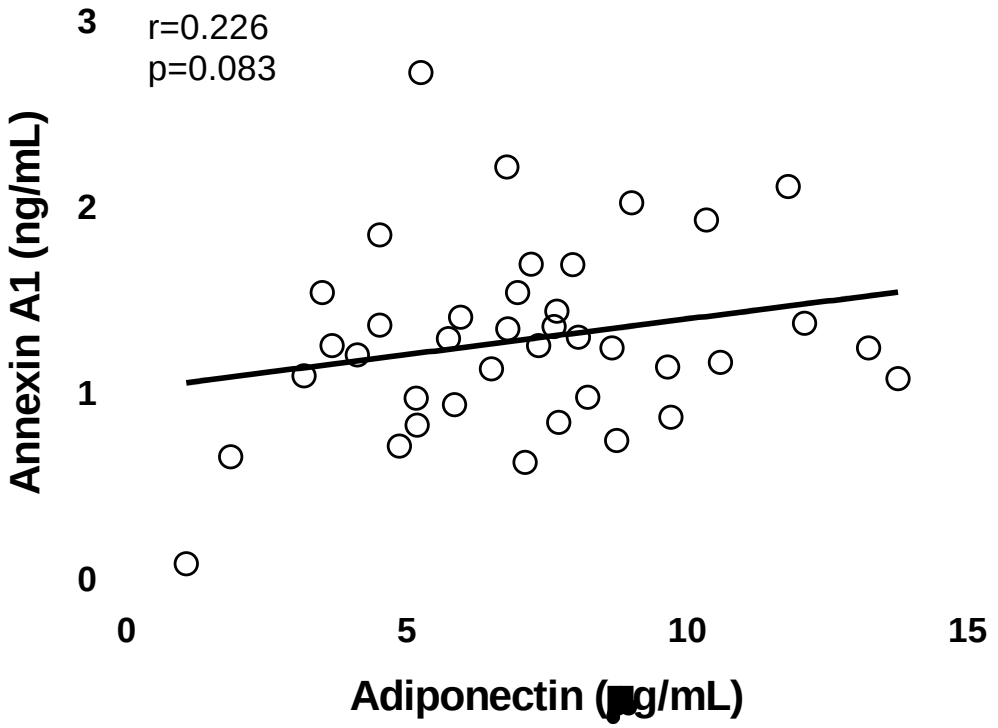


Figure 6.7: Correlation between plasma Annexin A1 levels and plasma Adiponectin levels in participant cohort. *Plasma Annexin A1 levels directly correlated with plasma Adiponectin levels. One tailed Person correlation coefficient was used to statistically analyse the data.*

6.5.2 Pre and post bariatric surgery: Surgery Type

Participant Characteristics: Surgery Type							
		Pre-Opt		6 Weeks Post-Opt		3 Months Post-Opt	
		SG	RYGB	SG	RYGB	SG	RYGB
Number of participants		13	13	7	9	5	6
Gender							
	Males	2	2	0	0	0	0
	Females	11	11	7	9	5	6
Age, years							
		51±2.3 (n=13)	45±2.9 (n=13)	49±4.1 (n=7)	44±3.3 (n=9)	54±2.4 (n=5)	48±4.2 (n=6)
Type 2 diabetes mellitus							
	Diabetics	5	5	3	4	2	2
	Non-Diabetics	8	8	4	5	3	4
Anthropometric Measures							
	Weight (kg)	136.1±5.3 (n=13)	138±5.4 (n=13)	117.5±7.0 (n=7)	125.8±8.0 (n=9)	104.6±7.6$^{\#}$ (n=5)	107.5±8.3* (n=4)
	BMI (kg/m^2)	49.9±1.8 (n=13)	49.5±1.5 (n=13)	43.3±2.4 (n=7)	45.8±2.1 (n=9)	38.8±2.1* (n=5)	38.8±2.5* (n=4)
Lipid Profiles							
	Triglycerides (mmol/L)	1.3±0.1 (n=12)	1.3±0.1 (n=13)	1.5±0.1 (n=7)	1.5±0.1 (n=8)	2.0±0.5 (n=4)	1.0±0.1$^{\#}$ (n=5)
	Total Cholesterol (mmol/L)	4.4±0.1 (n=12)	4.5±0.2 (n=13)	5.1±0.4 (n=7)	4.5±0.2 (n=8)	5.2±0.6 (n=4)	4.0±0.3 (n=5)
	HDL (mmol/L)	1.3±0.1 (n=12)	1.1±0.1 (n=13)	1.2±0.1 (n=7)	1.0±0.04 (n=8)	1.5±0.1 (n=4)	1.2±0.1 (n=5)
	LDL (mmol/L)	2.5±0.1 (n=12)	2.9±0.2 (n=13)	3.1±0.4 (n=7)	2.8±0.2 (n=8)	2.8±0.3 (n=4)	2.3±0.2 (n=5)

Table 6.3: Participant characteristics at pre- and post- Sleeve Gastrectomy and Roux-en-Y Gastric Bypass. *The anthropometric measures and lipid profiles were analysed in participants undergoing bariatric surgery at pre-, 6 weeks and 3 months post-operatively and compared between surgery types (SG vs RYGB). Two-way ANOVA followed by Tukey's Post-hoc multiple comparison test was used to statistically analyse the data. Data presented as Mean±SEM. P value was set at p <0.05 and is denoted by *vs pre opt, #vs 6 weeks of the corresponding group. Pre-opt= Pre-operatively, Post-opt= Post-operatively, SG= Sleeve gastrectomy, RYGB= Roux-en-Y Gastric Bypass, BMI= Body mass index, HDL= High density lipoprotein and LDL= Low density lipoprotein*

The characteristics of the SG and RYGB cohorts are presented in table 6.3. Of the 13 SG participants at pre-opt, 2 were male and 11 were female, with an average age of 51±2.3. At 6 weeks, 7 females (average age 49±4.1) and at 3 months, 5 females (average age 54±2.4) continued their participation. Similarly, of the 13 RYGB participants at pre-opt, 2 were males and 11 were females, with an average age of 45±2.9. At 6 weeks, 9 females (average age 44±3.3) and at 3 months, 6 females (average age 48±4.2) continued their participation.

6.5.2.1 Anthropometric and plasma lipid levels

The effect of time (p=0.001) but not surgery type (p=0.840) nor it's interaction with time (p=0.773) was statistically significant on the average weight loss. The effect of time (p=0.003) (but not surgery type (p=0.724) nor it's interaction with time (p=0.954)) was statistically significant on the average change in BMI. Furthermore, post-hoc analysis did not detect specific significant differences in the average weight and BMI between the cohorts at any time point.

However, the average weight decreased in both cohorts at post-opt compared to pre-opt. On average, in the SG cohort the average weight was significantly reduced at 3 months post-opt compared to 6 weeks post opt (104.6±7.6 kg, n=5 vs 117.5±7.0 kg, n=7, p=0.025 respectively) (Table 6.3). Similarly, in the RYGB the average weight was significantly reduced at 3 months post-opt compared to pre-opt (107±8.3 kg, n=4 vs 138±5.4 kg, n=13, p=0.040, respectively) (Table 6.3). Furthermore, the average BMI was reduced in both cohorts at post-opt compared to pre-opt. On average, in the SG cohort the average BMI was significantly reduced at 3 months post-opt compared to 6 weeks post-opt (39.0±2.1 kg/m^2, n=5 vs 43.3±2.4 kg/m^2, n=7, p=0.0247, respectively), as shown in Figure 6.8.

Similarly, in the RYGB cohort the average BMI was significantly reduced at 3 months post-opt compared to pre-opt (38.6±2.5 kg/m^2, n=4 vs 49.5±1.5 kg/m^2, n=13, p=0.039, respectively), as shown in Figure 6.8.

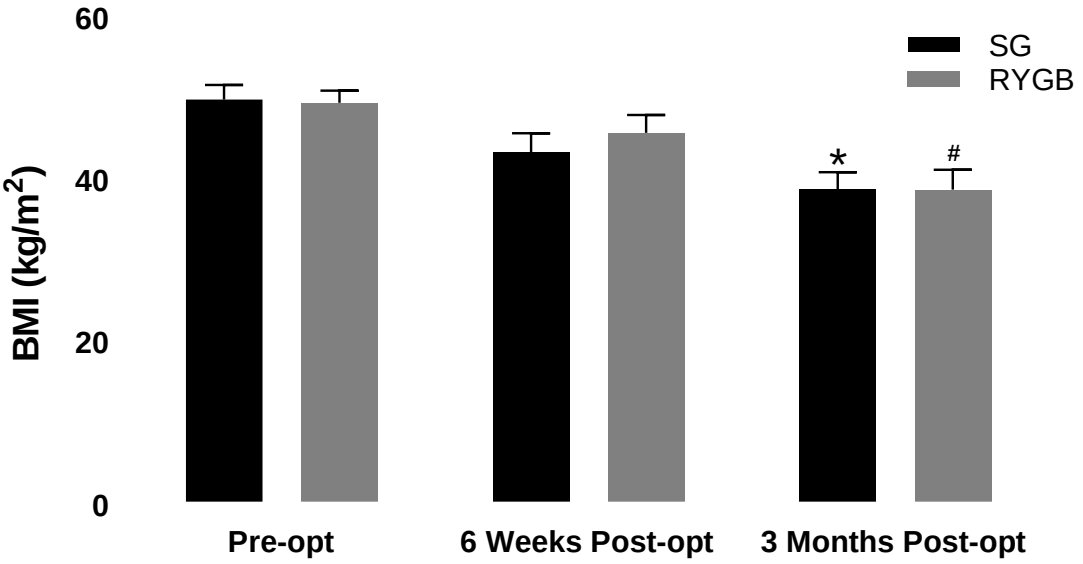


Figure 6.8: Average BMI (kg/m^2) of participant cohort pre- and post- Sleeve gastrectomy and Roux-en-Y Gastric Bypass. *The average BMI was significantly reduced at 3 months post-opt compared to 6 weeks post-opt in the SG cohort (p=0.037, n=13). Similarly, the average BMI was significantly reduced at 3 months post-opt compared to pre-opt in the RYGB cohort (p=0.038, n=13). However, no statistically significant differences were observed between the cohorts at any time point. Two-way ANOVA followed by Tukey's Post-hoc multiple comparison test was used to statistically analyse the data. Data presented as Mean±SEM. P value was set at p <0.05 and is denoted by *vs SG pre opt, #vs RYGB pre-opt of the corresponding group BMI= Body mass index, SG= Sleeve gastrectomy, RYGB= Roux-en-Y Gastric Bypass, Pre-opt= Pre-operatively, Post-opt= Post-operatively*

The effect of surgery type was statistically significant on the average plasma triglyceride levels (p=0.013), plasma total cholesterol levels (p=0.026) and plasma HDL levels (p=0.015), however, the post-hoc analysis did not detect specific significant differences between the cohorts at any time point. However, no significant difference was observed in the average plasma LDL levels in either cohorts at any timepoint.

6.5.2.2 Plasma inflammatory biomarkers, markers of insulin sensitivity and gut hormone

Table 6.4 presents the analysis of plasma inflammatory biomarkers, plasma markers of insulin sensitivity and plasma gut hormones pre- and post- SG and RYGB.

6.5.2.2.1 Plasma inflammatory biomarkers

The effect of time (p=0.184), surgery type (p=0.232) nor their interaction (p=0.429) was statistically significant on the average plasma CRP levels, therefore, no significant differences were observed between the two cohorts at any timepoint. However, the average plasma CRP levels decreased in both cohorts at post-opt compared to pre-opt. In the SG cohort, the average plasma CRP levels decreased at 6 weeks and 3 months post-opt compared to pre-opt, however, the difference observed was not statistically significant (10.6±3.7 µg/mL, n=7 and 9.5±4.2 µg/mL, n=5 vs 12.9±3.5 µg/mL, n=11, respectively). Similarly, in the RYGB cohort, the average plasma CRP levels decreased at 6 weeks and 3 months post-opt compared to pre-opt, however, the difference observed was not statistically significant (9.6±3.0 µg/mL, n=7 and 3.0±2.5 µg/mL, n=2 vs 9.6±2.8 µg/mL, n=7, respectively).

The effect of time (p=0.016) and surgery type (p=0.016) but not their interaction (p=0.864) was statistically significant on the average plasma IL-1β levels, however, the post-hoc analysis did not detect specific significant differences between the cohorts at any timepoint. However, the average plasma IL-1β levels increased in both cohorts at post-opt compared to pre-opt. In the SG cohort, the average plasma IL-1β levels increased at 3 months post-opt compared to pre-opt, however, the difference observed was not statistically significant (2.0±0.2 pg/mL, n=5 vs 1.0±0.2 pg/mL, n=10,

respectively). Similarly, in the RYGB cohort, the average plasma IL-1β levels increased at 6 weeks and 3 months post-opt compared to pre-opt, however, the difference observed was not statistically significant (1.6±0.2 pg/mL, n=9 and 2.5±0.6 pg/mL, n=4 vs 1.8±0.3 pg/mL, n=7, respectively).

The effect of time (p=0.056), surgery type (p=0.661) nor their interaction (p=0.940) was statistically significant on the average plasma IL-10 levels, therefore, no significant differences were observed between the two cohorts at any timepoint. However, the average plasma IL-10 levels increased in both cohorts at post-opt compared to pre-opt. In the SG cohort, the average plasma IL-10 levels increased at 6 weeks and 3 months post-opt compared to pre-opt, however, the difference observed was not statistically significant (9.5±1.0 pg/mL, n=6 and 9.0±0.6 pg/mL, n=5 vs 6.9±1.1 pg/mL, n=11, respectively). Similarly, in the RYGB cohort, the average plasma IL-10 levels increased at 6 weeks and 3 months post-opt compared to pre-opt, however, the difference observed was not statistically significant (10.1±1.2 pg/mL, n=8 and 9.1±2.0 pg/mL, n=3 vs 6.9±0.7 pg/mL, n=7, respectively).

The effect of time (p=0.914), surgery type (p=0.653) nor their interaction (p=0.748) was statistically significant on the average plasma TNFα levels, therefore, no significant differences were observed between the two cohorts at any timepoint. However, the average plasma TNFα levels remained similar in both cohorts at post-opt compared to pre-opt. In the SG cohort, the average plasma TNFα levels remained similar at 6 weeks and 3 months post-opt (22.0±1.3 pg/mL, n=6 and 21.8±2.1 pg/mL, n=5 vs 21.9±0.6 pg/mL, n=11, respectively). Similarly, in the RYGB cohort, the average plasma TNFα levels remained similar at 6 weeks and 3 months post-opt compared to pre-opt (20.6±1.1 pg/mL, n=9 and 21.6±1.8 pg/mL, n=4 vs 21.4±0.5 pg/mL, n=8, respectively).

The effect of time (p=0.001) but not the surgery type (p=0.994) nor their interaction (p=0.551) was statistically significant on the average plasma leptin levels, however, the post-hoc analysis did not detect specific significant differences between the cohorts at any timepoint. However, the average plasma leptin levels decreased in both cohorts at post-opt compared to pre-opt. In the SG cohort, the average plasma leptin levels significantly decreased at 6 weeks and 3 months post-opt, however, the difference observed was not statistically significant (35.0±6.3 ng/mL, n=7 and 32.8±8.4 ng/mL, n=5 vs 85.3±9.1 ng/mL, n=12, respectively). A similar pattern was observed in the RYGB cohort, the average plasma leptin levels significantly decreased at 6 weeks post-opt compared to pre-opt, however, the difference observed was not statistically significant (36.1±7.4 ng/mL, n=9 vs 74.3±10.5 ng/mL, n=8, p=0.004, respectively) and at 3 months post-opt compared to pre-opt (39.2±4.0 ng/mL, n=4 and 74.3±10.5 ng/mL, n=8, respectively).

The effect of time (p=0.015) but not the surgery type (p=0.397) nor their interaction (p=0.257) was statistically significant on the average plasma adiponectin levels, however, the post-hoc analysis did not detect specific significant differences between the cohorts at any timepoint. However, the average plasma adiponectin levels increased in both cohorts at post-opt compared to pre-opt. In the SG cohort, the average plasma adiponectin levels increased at 6 weeks and 3 months post-opt compared to pre-opt, however, the difference observed was not statistically significant (8.9±1.6 µg/mL, n=6 and 8.0±1.1 µg/mL, n=5 vs 5.7±0.8 µg/mL, n=11, respectively). Similarly, in the RYGB cohort, the average plasma adiponectin levels increased at 6 weeks and 3 months post-opt compared to pre-opt, however, the difference observed was not statistically significant (7.6±1.2 µg/mL, n=9 and 10.5±0.8 µg/mL, n=3 vs 6.0±0.7 µg/mL, n=6, respectively).

6.5.2.2.2 Plasma biomarkers of insulin sensitivity

The effect of time (p=0.933), surgery type (p=0.878) nor their interaction (p=0.765) was statistically significant on the average fasting plasma glucose levels, therefore, no significant differences were observed between the two cohorts at any timepoint. However, the average fasting plasma glucose levels remained similar in both cohorts at post-opt compared to pre-opt. In the SG cohort, the average fasting plasma glucose levels remained similar at 6 weeks and 3 months post-opt compared to pre-opt (4.1±0.4 mmol/L, n=5 and 3.8±0.1 mmol/L, n=3 vs 4.1±0.2 mmol/L, n=11, respectively). Similarly, in the RYGB cohort, the average fasting plasma glucose levels remained similar at 6 weeks and 3 months post-opt compared to pre-opt (4.0±0.2 mmol/L, n=4 and 4.4±0.5 mmol/L, n=4 vs 3.9±0.8 mmol/L, n=8, respectively).

The effect of time (p=0.063), surgery type (p=0.126) nor their interaction (p=0.563) was statistically significant on the average fasting plasma insulin levels, therefore, no significant differences were observed between the two cohorts at any timepoint. However, the average fasting plasma insulin levels decreased in both cohorts at post-opt compared to pre-opt. In the SG cohort, the average fasting plasma insulin levels significantly decreased at 6 weeks compared to pre-opt, however, the difference observed was not statistically significant (18.2±3.5 µlU/mL, n=5 vs 29.2±2.9 µlU/mL, n=11, p=0.032, respectively). Similarly, in the RYGB cohort, the average fasting plasma insulin levels decreased at 6 weeks and 3 months post-opt compared to pre-opt, however, the difference observed was not statistically significant (18.0±0.9 µlU/mL, n=4 and 13.8±2.8 µlU/mL, n=4 vs 21.0±1.4 µlU/mL, n=8, respectively).

The effect of surgery type (p=0.007) but not time (p=0.553) nor their interaction (p=0.723) was statistically significant on the average HOMA-IR, however, the post-hoc analysis did not detect specific significant differences between the cohorts at any timepoint. However, the average HOMA-IR remained similar in both at post-opt compared to pre-opt. In the SG cohort, the average HOMA-IR remained similar at 6 weeks and 3 months post-opt compared to pre-opt (3.6±0.4, n=6 and 3.3±0.7, n=3 vs 3.6±0.4, n=9, respectively). Similarly, in the RYGB cohort, the average HOMA-IR remained similar at 6 weeks and 3 months post-opt compared to pre-opt (2.8±0.6, n=2 and 2.7±0.3, n=4 vs 2.6±0.2, n=6, respectively).

The effect of time (p=0.492), surgery type (p=0.054) nor their interaction (p=0.969) was statistically significant on the average HOMA-%S, therefore, no significant differences were observed between the two cohorts at any timepoint. However, the average HOMA-%S remained similar in both cohorts post-opt compared to pre-opt. In the SG cohort, the average HOMA-%S remained similar at 6 weeks and 3 months post-opt compared to pre-opt (30.3±4.4 %, n=6 and 33.7±8.8 %, n=3 vs 30.8±3.5 %, n=9, respectively). Similarly, in the RYGB cohort, the average HOMA-%S remained similar at 6 weeks and 3 months post-opt compared to pre-opt (2.8±0.6 %, n=2 and 2.7±0.3 %, n=4 vs 2.6±0.2 %, n=6, respectively).

The effect of time (p=0.285), surgery type (p=0.289) nor their interaction (p=0.879) was statistically significant on the average HOMA-%β, therefore, no significant differences were observed between the two cohorts at any timepoint. However, the average HOMA-%β reduced in both cohorts at post-opt compared to pre-opt. In the SG cohort, the average HOMA-%β reduced at 6 weeks and 3 months post-opt compared to pre-opt, however, the difference observed was not statistically significant (267.2±16.2 %, n=5 and 252.2±42.2

%, n=3 vs 326.4±42.4 %, n=9, respectively). Similarly, in the RYGB cohort, the average HOMA-%β reduced at 6 weeks and 3 months post-opt compared to pre-opt, however, the difference observed was not statistically significant (251.9±13.6 %, n=4 and 204.2±53.2 %, n=4 vs 269.7±42.2 %, n=6, respectively).

6.5.2.2.3 Plasma gut hormone

The effect of time (p=0.001) but not the surgery type (p=0.998) nor their interaction (p=0.496) was statistically significant on the average plasma GLP-1 levels, however, the post-hoc analysis did not detect specific significant differences between the cohorts at any timepoint. However, the average plasma GLP-1 levels increased in both cohorts at post-opt compared to pre-opt. In the SG cohort, the average plasma GLP-1 levels increased at 6 weeks (308.2±24.1 ng/mL, n=5 vs 259.7±17.4 ng/mL, n=11, respectively) and significantly 3 months post-opt compared to pre-opt (376.8±17.7 ng/mL, n=3 vs 259.7±17.4 ng/mL, n=11, $p<0.01$) respectively). Similarly, in the RYGB cohort, the average plasma GLP-1 levels increased at 6 weeks and 3 months post-opt compared to pre-opt, however, the difference observed was not statistically significant (315.0±23.9 ng/mL, n=4 and 337.7±26.6 ng/mL, n=4 vs 270.3±16.8 ng/mL, n=8, respectively).

Plasma Biomarkers: Surgery Type							
		Pre-opt		6 Weeks Post-opt		3 Months Post-opt	
Surgery Type		SG	RYGB	SG	RYGB	SG	RYGB
Inflammatory Biomarkers							
	CRP (µg/mL)	12.9±3.5 (n=11)	9.6±2.8 (n=7)	10.6±3.7 (n=7)	9.6±3.0 (n=7)	9.5±4.2 (n=5)	3.0±2.5 (n=2)
	Annexin A1 (ng/mL)	1.1±0.1 (n=12)	1.1±0.1 (n=8)	1.7±0.3 (n=7)	1.2±0.1 (n=9)	1.6±0.2 (n=5)	1.6±0.3 (n=4)
	IL-1β (pg/mL)	1.0±0.2 (n=10)	1.8±0.3 (n=7)	1.1±0.2 (n=6)	1.6±0.2 (n=9)	2.0±0.2 (n=5)	2.5±0.6 (n=4)
	IL-10 (pg/mL)	6.9±1.1 (n=11)	6.9±0.7 (n=7)	9.5±1.0 (n=6)	10.1±1.2 (n=8)	9.0±0.6 (n=5)	9.1±2.0 (n=3)
	TNFα (pg/mL)	21.9±0.6 (n=11)	21.4±0.5 (n=8)	22.0±1.3 (n=6)	20.6±1.1 (n=9)	21.8±2.1 (n=5)	21.6±1.8 (n=4)
	Leptin (ng/mL)	85.3±9.1 (n=12)	74.3±10.5 (n=8)	35.0±6.3 (n=7)	36.1±7.4** (n=9)	32.8±8.4 (n=5)	39.2±4.0 (n=4)
	Adiponectin (µg/mL)	5.7±0.8 (n=11)	6.0±0.7 (n=6)	8.9±1.6 (n=6)	7.6±1.2 (n=9)	8.0±1.1 (n=5)	10.5±0.8 (n=3)
Insulin Sensitivity							
	Fasting Glucose (mmol/L)	4.1±0.2 (n=11)	3.9±0.8 (n=8)	4.1±0.4 (n=5)	4.0±0.2 (n=4)	3.8±0.1 (n=3)	4.4±0.5 (n=4)
	Fasting Insulin (µIU/mL)	29.2±2.9 (n=11)	21.0±1.4 (n=8)	18.2±3.5* (n=5)	18.0±0.9 (n=4)	15.8±5.0 (n=3)	13.8±2.8 (n=4)
	HOMA-IR	3.6±0.4 (n=9)	2.6±0.2 (n=6)	3.6±0.4 (n=6)	2.8±0.6 (n=2)	3.3±0.7 (n=3)	2.7±0.3 (n=4)
	HOMA-%S (%)	30.8±3.5 (n=9)		30.3±4.4 (n=6)		33.7±8.8 (n=3)	
	HOMA-%β (%)	326.4±42.2 (n=9)	269.7±42.2 (n=6)	267.2±16.2 (n=2)	251.9±13.6 (n=4)	252.2±42.2 (n=3)	204.2±53.2 (n=4)
Gut Hormone							
	GLP-1 (ng/mL)	259.7±17.4 (n=11)	270.3±16.8 (n=8)	308.2±24.1 (n=5)	315.0±23.9 (n=4)	376.8±17.7** (n=3)	337.7±26.6 (n=4)

Table 6.4: Plasma biomarkers analysis of participant cohort at pre- and post- Sleeve Gastrectomy and Roux-en-Y Gastric Bypass. *Plasma inflammatory biomarkers, fasting plasma glucose and insulin levels and plasma gut hormone were analysed and, HOMA-IR, HOMA-%S and HOMA-%β were calculated using the equations presented in section 6.4.2.2. One-way ANOVA followed by Tukey's Post-hoc multiple comparison test was used to statistically analyse the data. Data presented as Mean±SEM.*
*P value was set at p <0.05 and is denoted by *vs pre opt of the corresponding group*
Pre-opt= Pre-operatively, Post-opt= Post-operatively, SG= Sleeve gastrectomy, RYGB= Roux-en-Y Gastric Bypass, CRP= C-reactive protein, IL-= Interleukin, TNFα= Tumour necrosis factor α, HOMA-IR= Homeostatic model assessment of insulin resistance, HOMA-%S= Homeostatic model assessment of insulin sensitivity, HOMA-%β= Homeostatic model assessment of pancreatic β cell function and GLP-1= Glucagon like peptide 1

6.5.2.3 Annexin A1

The effect of time (p=0.058), surgery type (p=0.321) nor their interaction (p=0.283) was statistically significant on the average plasma ANXA1 levels, therefore, no significant differences were observed between the two cohorts at any timepoint. However, plasma ANXA1 levels increased in both cohorts at post-opt compared to pre-opt. In the SG cohort, the average plasma ANXA1 levels increased at 6 weeks and 3 months post-opt compared to pre-opt, however, the difference observed was not statistically significant (1.7±0.3 ng/mL, n=7 and 1.6±0.2 ng/mL, n=5 vs 1.1±0.1 ng/mL, n=12, respectively), as shown in Figure 6.9. Similarly, in the RYGB cohort, the average plasma ANXA1 levels increased at 6 weeks and 3 months post-opt compared to pre-opt, however, the difference observed was not statistically significant (1.2±0.1 ng/mL, n=9 and 1.6±0.3 ng/mL, n=4 vs 1.1±0.1 ng/mL, n=8, respectively), as shown in Figure 6.9.

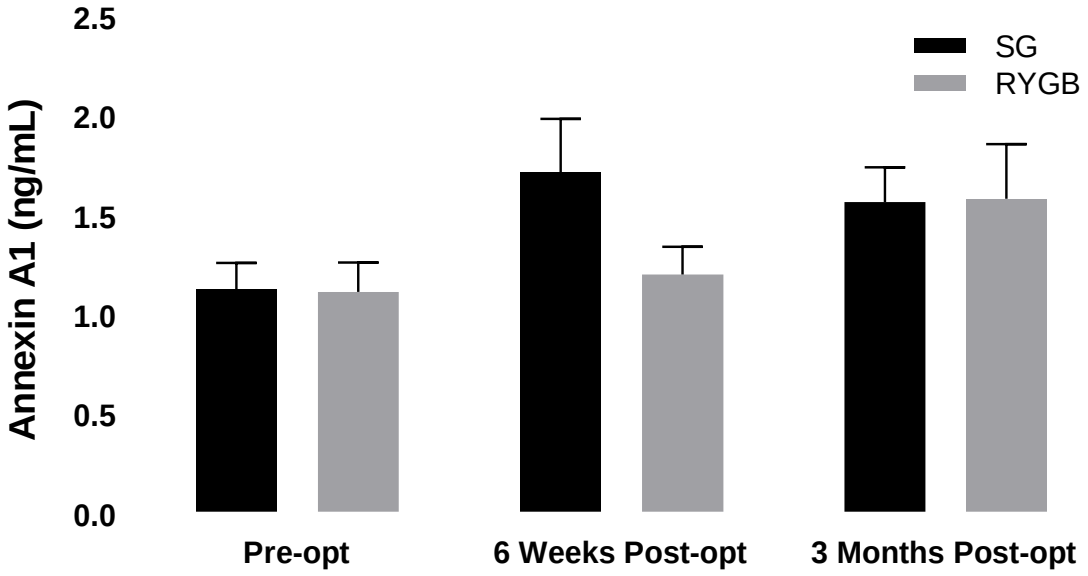


Figure 6.9: Average plasma Annexin A1 (ng/mL) levels of participant cohort pre- and post- Sleeve Gastrectomy and Roux-en-Y Gastric Bypass. *The average plasma Annexin A1 levels increased at 3 months post-opt compared to 6 weeks post-opt and pre-opt in both cohorts, however, no statistically significant difference was observed at any time point within and between cohorts. Two-way ANOVA followed by Tukey's Post-hoc multiple comparison test was used to statistically analyse the data. Data presented as Mean±SEM. P value was set at p <0.05.*
SG= Sleeve gastrectomy, RYGB= Roux-en-Y Gastric Bypass, Pre-opt= Pre-operatively, Post-opt= Post-operatively

6.5.2.3.1 Annexin A1: SG

Plasma ANXA1 levels significantly inversely correlated with BMI (r=-0.389, p=0.030, n=24) (Figure 6.10) and significantly directly correlated with plasma adiponectin levels (r=0.440, p=0.020, n=22) (Figure 6.11) in the SG cohort.

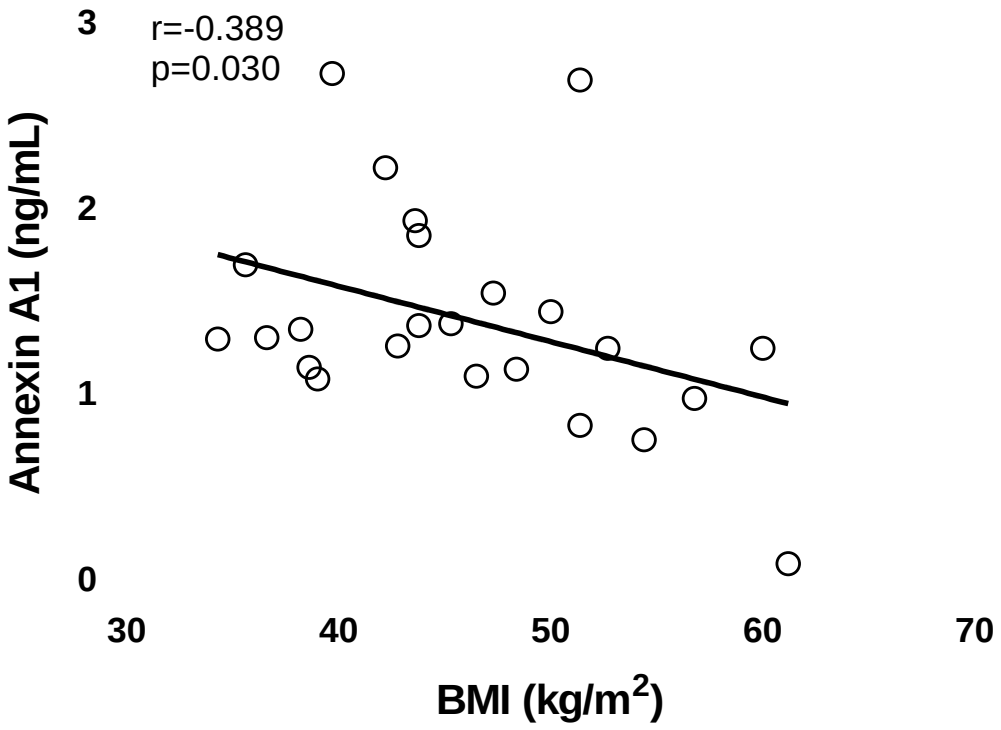


Figure 6.10: Correlation between plasma Annexin A1 levels and BMI in Sleeve Gastrectomy participant cohort. *Plasma Annexin A1 levels significantly inversely correlated with BMI. One tailed Person correlation coefficient was used to statistically analyse the data.*
BMI= Body mass index

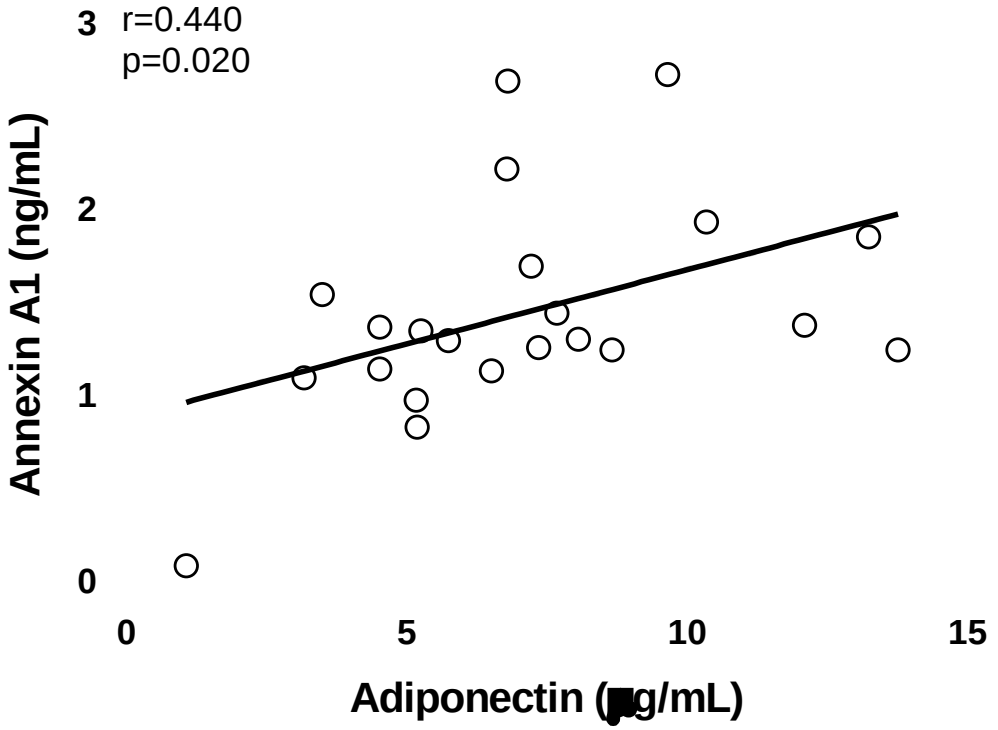


Figure 6.11: Correlation between plasma Annexin A1 levels and plasma Adiponectin levels in Sleeve Gastrectomy participant cohort. *Plasma Adiponectin levels significantly directly correlated with BMI. One tailed Person correlation coefficient was used to statistically analyse the data.*

6.5.2.3.2 Annexin A1: RYGB

Plasma ANXA1 levels significantly inversely correlated with plasma triglyceride levels (r=-0.421, p=0.032, n=20) (Figure 6.12), directly correlated with plasma HDL levels (r=0.364, p=0.058, n=20) (Figure 6.13), significantly directly correlated with plasma CRP levels (r=0.518, p=0.020, n=16) (Figure 6.14), significantly directly with plasma IL-1β levels (r=0.464, p=0.026, n=18) (Figure 6.15), directly with plasma TNFα levels (r=0.363, p=0.053, n=21) (Figure 6.16) and significantly directly with plasma GLP-1 levels (r=0.689, p=0.002, n=16) (Figure 6.17) in the RYGB cohort.

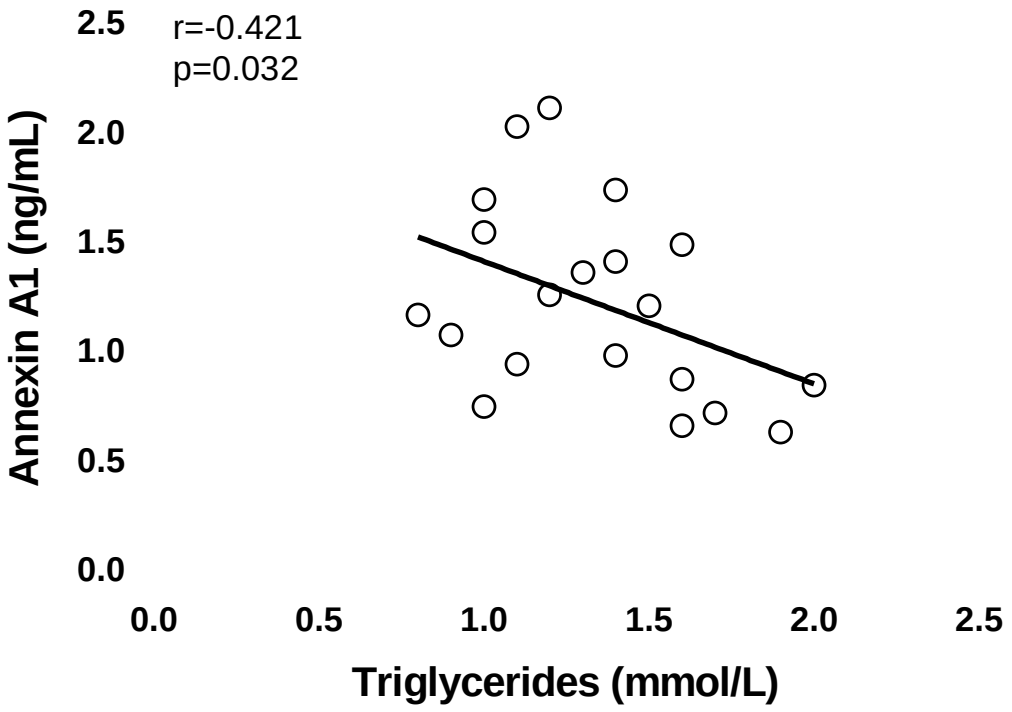


Figure 6.12: Correlation between plasma Annexin A1 levels and plasma Triglyceride levels in Roux-en-Y Gastric Bypass participant cohort. *Plasma Annexin A1 levels significantly inversely correlated with plasma triglyceride levels. One tailed Person correlation coefficient was used to statistically analyse the*

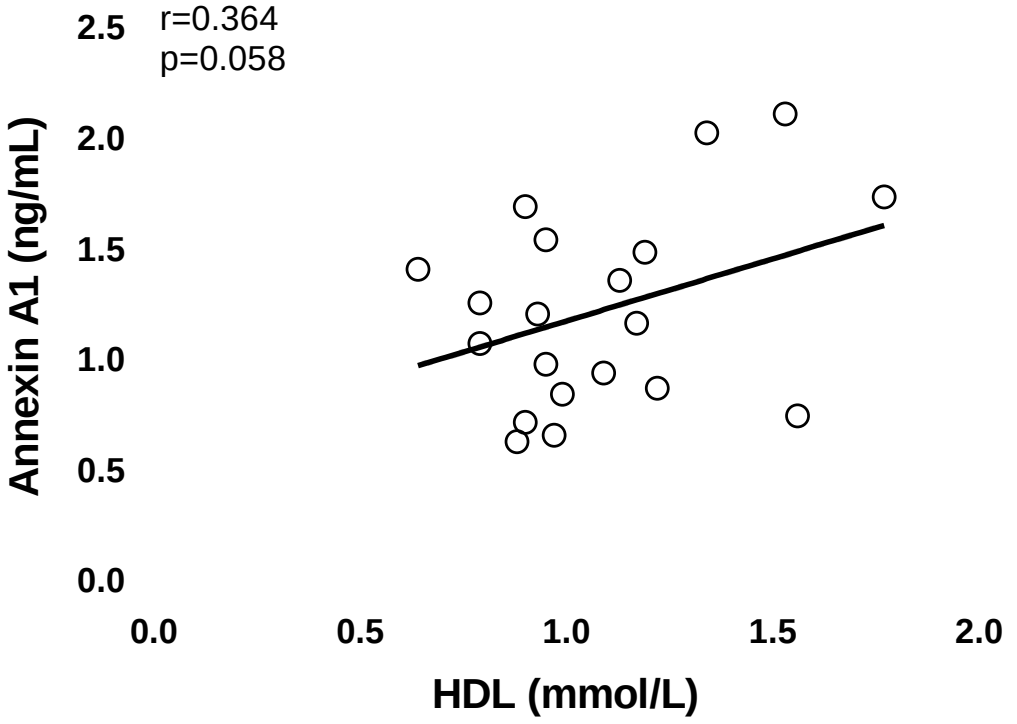


Figure 6.13: Correlation between plasma Annexin A1 levels and plasma HDL levels in Roux-en-Y Gastric Bypass participant cohort. *Plasma Annexin A1 levels directly correlated with plasma HDL levels. One tailed Person correlation coefficient was used to statistically analyse the data.*
HDL= High density lipoprotein

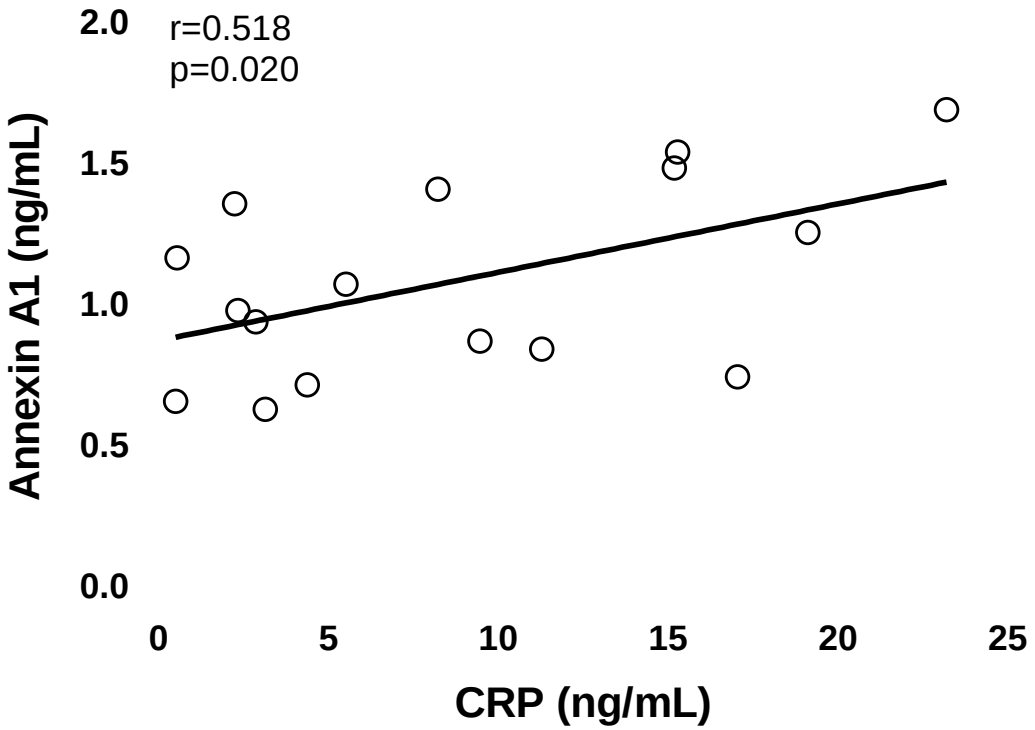


Figure 6.14: Correlation between plasma Annexin A1 levels and plasma CRP levels in Roux-en-Y Gastric Bypass participant cohort. *Plasma Annexin A1 levels significantly directly correlated with plasma CRP levels. One tailed Person correlation coefficient was used to statistically analyse the data.*
CRP= C-reactive peptide

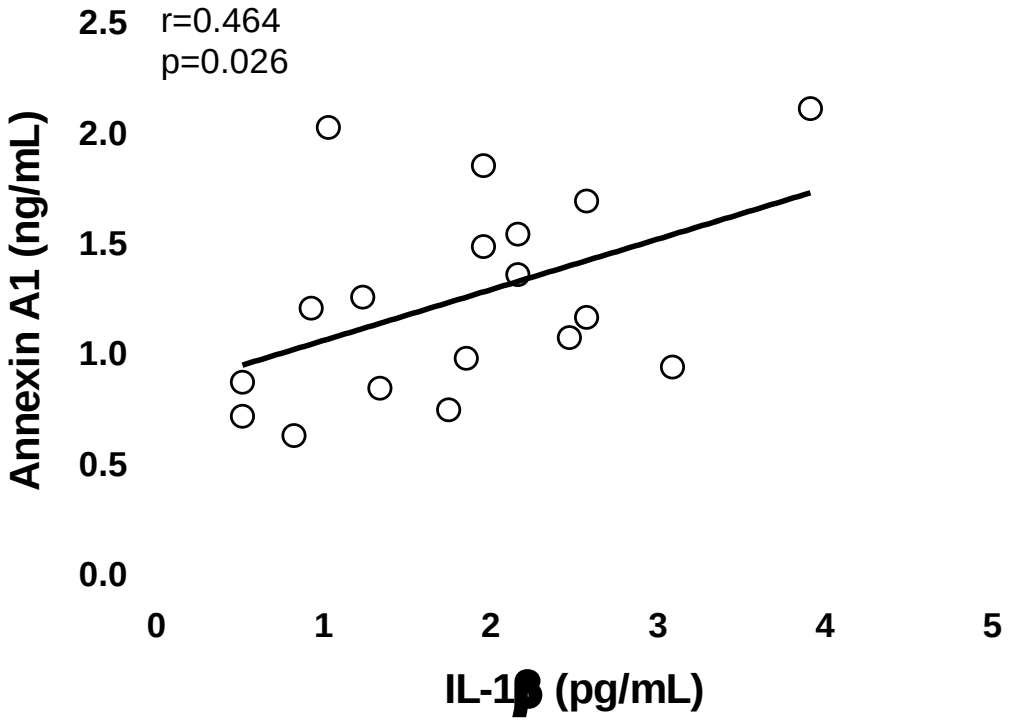


Figure 6.15: Correlation between plasma Annexin A1 levels and plasma IL-1β levels in Roux-en-Y Gastric Bypass participant cohort. *Plasma Annexin A1 levels significantly directly correlated with plasma IL-1β levels. One tailed Person correlation coefficient was used to statistically analyse the data.*
IL-1β= interleukin-1β

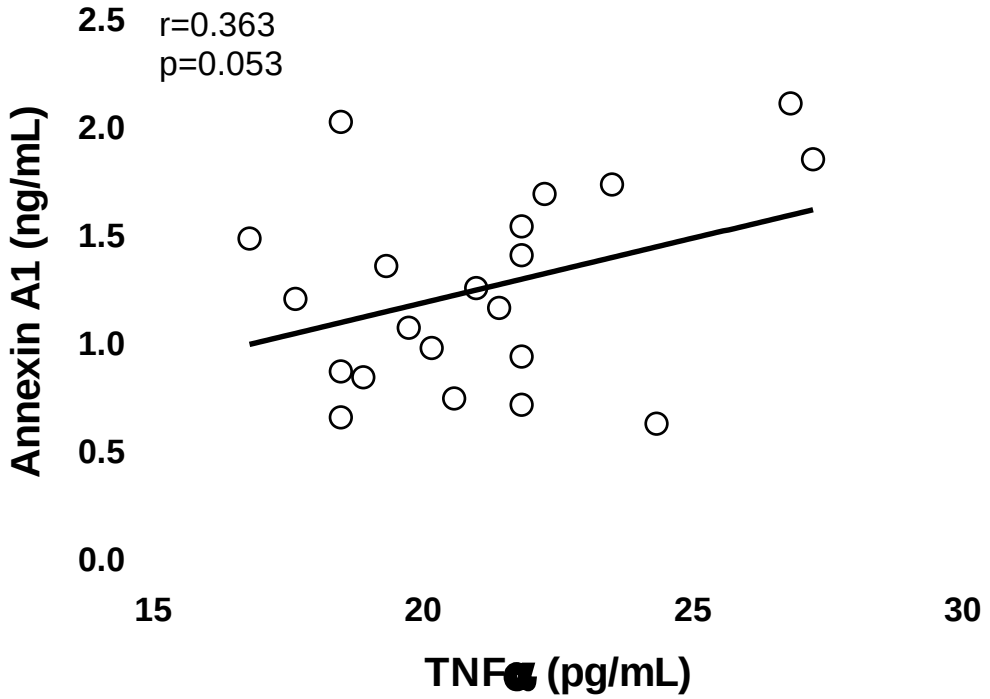


Figure 6.16: Correlation between plasma Annexin A1 levels and plasma TNF α levels in Roux-en-Y Gastric Bypass participant cohort. *Plasma Annexin A1 levels directly correlated with plasma TNFα levels. One tailed Person correlation coefficient was used to statistically analyse the data.*
TNFα= Tumour necrosis factor α

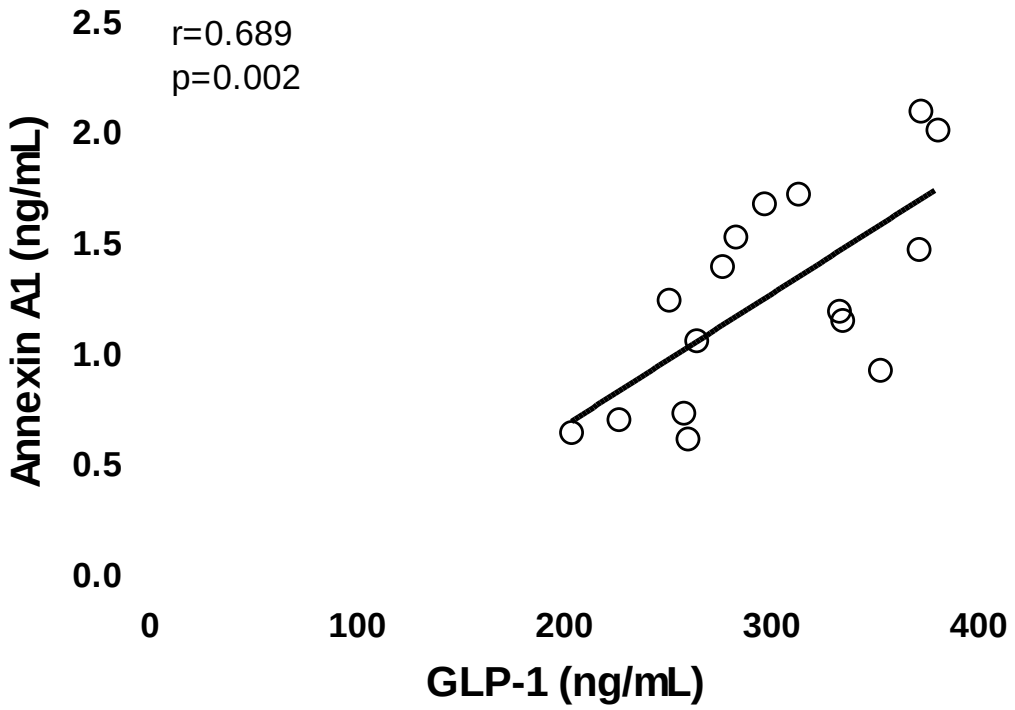


Figure 6.17: Correlation between plasma Annexin A1 levels and plasma GLP-1 levels in Roux-en-Y Gastric Bypass participant cohort. *Plasma Annexin A1 levels significantly directly correlated with plasma GLP-1 levels. One tailed Person correlation coefficient was used to statistically analyse the data.*
GLP-1= Glucagon like peptide- 1

6.5.3 Pre and post bariatric surgery: Type 2 diabetes mellitus Status

Participant Characteristics: Type 2 diabetes mellitus status							
		Pre-Opt		6 Weeks Post-Opt		3 Months Post-Opt	
		Non-T2DM	T2DM	Non-T2DM	T2DM	Non-T2DM	T2DM
Number of participants		16	10	9	7	7	4
Gender							
	Males	3	1	0	0	0	0
	Females	13	9	9	7	7	4
Age, years							
		50±2.4 (n=16)	45±2.9 (n=10)	48±3.7 (n=9)	43±3.5 (n=7)	53±2.8 (n=7)	47±5.6 (n=4)
Type of Surgery							
	Sleeve Gastrectomy	8	5	4	3	3	2
	Roux-en-Y Gastric Bypass	8	5	5	4	4	2
Anthropometric Measures							
	Weight (kg)	125.1±9.4 (n=16)	141.7±6.3 (n=10)	115.4±6.2 (n=9)	131.7±9.3 (n=6)	110.9±6.0 (n=5)	99.65±9.1** (n=4)
	BMI (kg/m^2)	48.5±1.4 (n=16)	51.6±2.0 (n=10)	42.1±1.6* (n=9)	48.5±2.5 (n=6)	39.8±1.9** (n=5)	37.6±2.6***/## (n=4)
Lipid Profiles							
	Triglycerides (mmol/L)	1.3±0.1 (n=15)	1.3±0.1 (n=10)	1.4±0.1 (n=9)	1.6±0.1 (n=7)	1.3±0.2 (n=5)	2.6±0.4 (n=4)
	Total Cholesterol (mmol/L)	4.6±1.2 (n=15)	4.3±0.2 (n=10)	4.6±0.4 (n=9)	4.9±0.3 (n=7)	4.3±0.3 (n=5)	4.8±0.7 (n=4)
	HDL (mmol/L)	1.1±0.1 (n=15)	1.2±0.1 (n=10)	1.1±0.1 (n=9)	1.1±0.1 (n=7)	1.3±0.1 (n=5)	1.4±0.1 (n=4)
	LDL (mmol/L)	2.8±0.1 (n=15)	2.5±0.2 (n=10)	2.8±0.3 (n=9)	3.1±0.2 (n=7)	2.4±0.2 (n=5)	1.6±0.6 (n=4)

Table 6.5: Participant characteristics of non-T2DM and T2DM cohort pre- and post- Bariatric Surgery. *The anthropometric measures and lipid profiles were analysed in participants undergoing bariatric surgery at pre-, 6 weeks and 3 months post-operatively and compared between surgery types. Two-way ANOVA followed by Tukey's Post-hoc multiple comparison test was used to statistically analyse the data. Data presented as Mean±SEM. P value was set at p <0.05 and is denoted by *vs pre opt of the corresponding group. Pre-opt= Pre-operatively, Post-opt= Post-operatively, T2DM= Type 2 diabetes mellitus, BMI= Body mass index, HDL= High density lipoprotein and LDL= Low density lipoprotein*

The characteristics of the non-T2DM and T2DM cohorts are presented in table 6.5. Of the 16 non-T2DM participants at pre-opt, 3 were male and 13 were female, with an average age of 50±2.4. At 6 weeks, 9 females (average age 48±3.7) and at 3 months, 7 females (average age 53±2.8) continued their participation. Similarly, of the 10 T2DM participants at pre-opt, 1 was male and 9 were females, with an average age of 45±2.9. At 6 weeks, 7 females (average age 43±3.5) and at 3 months, 4 females (average age 47±5.6) continued their participation.

6.5 .3.1 Anthropometric and plasma lipid levels

The effect of time (p=0.060), T2DM status (p=0.400) nor their interaction (p=0.391) was statistically significant on the average weight. The effect of time (p=0.0001) (but not T2DM status (p=0.167) nor it's interaction with time (p=0.196)) was statistically significant on the average change in BMI. Therefore, no significant differences in the average weight and BMI were observed between the two cohorts at any timepoint.

However, the average weight decreased in both cohorts at post-opt compared to pre-opt. In the non-T2DM cohort, the average weight was reduced at 6 weeks and 3 months post-opt compared to pre-op, however, the difference observed was not statistically significant (115.4±6.2 kg, n=9 and 110.9±6.0 kg, n=5 vs 125.1±9.4 kg, n=16, respectively) (Table 6.5). Similarly, in the T2DM cohort, the average weight was reduced at 3 months post-opt compared to pre-opt, however, the difference observed was not statistically significant (99.7±9.1 kg, n=4 vs 141.7±6.3 kg, n=10, p=0.008, respectively) (Table 6.5). Furthermore, the average BMI was reduced in both cohorts at post-opt compared to pre-opt. In the non-T2DM cohort, the average BMI was significantly reduced at 6 weeks and 3 months post-opt compared to pre-opt, (42.1±1.6 kg/m^2, n=9, p=0.029 and 39.8±1.9

kg/m^2, n=5, p=0.009 vs 48.5±1.3 kg/m^2, n=16, respectively), as shown in Figure 6.18. Similarly in the T2DM cohort, the average BMI was reduced at 3 months post-opt compared to pre-opt (37.6±2.6 kg/m^2, n=4 vs 51.6±2.0 kg/m^2, n=10, p=0.0003, respectively) and to 6 weeks post-opt (48.5±2.5 kg/m^2, n=6 vs37.6±2.6 kg/m^2, n=4, p=0.009, respectively), as shown in Figure 6.18.

The effect of time, T2DM status nor their interaction was significantly significant on the average plasma triglyceride levels (p=0.323, p=0.235 and p=0.688, respectively), plasma total cholesterol levels (p=0.447, p=0.464 and p=0.339, respectively), plasma HDL levels (p=0.181, p=0.537 and p=0.701, respectively) and plasma LDL levels (p=0.248, p=0.857 and p=0.242, respectively), therefore, the post-hoc analysis did not detect specific significant differences between the cohorts at any time point.

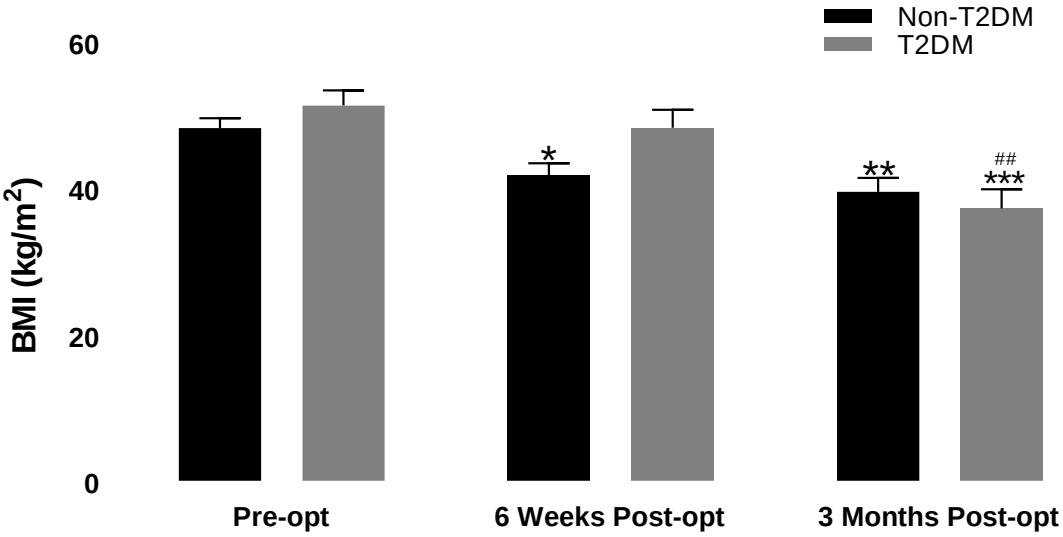


Figure 6.18: Average BMI (kg/m^2) of non-T2DM and T2DM cohort pre- and post- Bariatric surgery. *The average BMI was significantly reduced at 6 weeks and 3 months post-opt compared to pre-opt in the non-T2DM cohort (p=0.029, n=9 and p=0.009, n=5, respectively). Similarly, the average BMI was reduced at 3 months post-opt compared to pre-opt (p=0.0003, n=10) and to 6 weeks post opt (p=0.009, n=4) in T2DM cohort. However, no statistically significant differences were observed between the cohorts at any time point. Two-way ANOVA followed by Tukey's Post-hoc multiple comparison test was used to statistically analyse the data. Data presented as Mean±SEM.*
*P value was set at p <0.05 and is denoted by *vs pre opt and $^{\#}$vs 6 weeks post-opt of the corresponding group BMI= Body mass index, T2DM= Type 2 diabetes mellitus, Pre-opt= Pre-operatively, Post-opt= Post-operatively*

6.5.3.2 Plasma inflammatory biomarkers, markers of insulin sensitivity and gut hormones

Table 6.6 presents the analysis of plasma inflammatory biomarkers, plasma markers of insulin sensitivity and plasma gut hormones in non-T2DM and T2DM cohorts pre and post- bariatric surgery.

6.5.3.2.1 Plasma inflammatory biomarkers

The effect of time (p=0.657), T2DM status (p=0.540) nor their interaction (p=0.706) was statistically significant on the average plasma CRP levels, therefore, no significant differences were observed between the two cohorts at any timepoint. However, the average plasma CRP levels decreased in both cohorts at post-opt compared to pre-opt. In the non-T2DM cohort, the average plasma CRP levels decreased at 6 weeks and 3 months post-opt compared to pre-opt, however, the difference observed was not statistically significant (8.8±2.8 µg/mL, n=7 and 5.4±1.9 µg/mL, n=4 vs 12.1±2.0 µg/mL, n=12, respectively). Similarly in the T2DM cohort, the average plasma CRP levels decreased at 6 weeks and 3 months post-opt compared to pre-opt, however, the difference observed was not statistically significant (11.3±3.8 µg/mL, n=7 and 10.8±7.3 µg/mL, n=3 vs 10.6±6.3 µg/mL, n=6, respectively).

The effect of time (p=0.044) but not T2DM status (p=0.806) nor its interaction with time (p=0.732) was statistically significant on the average plasma IL-1β levels, however, the post-hoc analysis did not detect specific significant differences between the cohorts at any time point. However, the average plasma IL-1β levels increased in both cohorts at post-opt compared to pre-opt. In the non-T2DM cohort, the average plasma IL-1β levels increased at 3 months post-opt compared to pre-opt, however, the difference observed

was not statistically significant (2.3±0.4 pg/mL, n=5 vs 1.2±0.2 pg/mL, n=12, respectively). Similarly in the T2DM cohort, the average plasma IL-1β levels increased at 6 weeks and 3 months post-opt compared to pre-opt, however, the difference observed was not statistically significant (1.5±0.3 pg/mL, n=4 and 2.1±0.4 pg/mL, n=4 vs 1.5±0.5 pg/mL, n=5, respectively).

The effect of time (p=0.001) but not T2DM status (p=0.459) nor its interaction with time (p=0.544) was statistically significant on the average plasma IL-10 levels, however, the post-hoc analysis did not detect specific significant differences between the cohorts at any time point. However, the average plasma IL-10 levels increased in both cohorts at post-opt compared to pre-opt. In the non-T2DM cohort, the average plasma IL-10 levels significantly increased at 6 weeks and 3 months post-opt compared to pre-opt (10.0±1.2 pg/mL, n=8, p=0.021 and 9.3±0.9 pg/mL, n=4 vs 7.2±1.0 pg/mL, n=12, respectively). Similarly in the T2DM cohort, the average plasma IL-10 levels increased at 6 weeks and 3 months post-opt compared to pre-opt, however, the difference observed was not statistically significant (9.6±1.0 pg/mL, n=6 and 8.8±1.4 pg/mL, n=4 vs 6.4±0.8 pg/mL, n=6, respectively).

The effect of time (p=0.789), T2DM status (p=0.915) nor their interaction (p=0.699) was statistically significant on the average plasma TNFα levels, therefore, no significant differences were observed between the two cohorts at any time point. The average plasma TNFα levels remained similar in both cohorts at post-opt compared to pre-opt. In the non-T2DM cohort, the average plasma TNFα levels remained similar at 6 weeks and 3 months post-opt (21.5±1.1 pg/mL, n=9 and 21.2±1.5 pg/mL, n=5 vs 21.7±0.4 pg/mL, n=14, respectively). Similarly, in the T2DM cohort, the average plasma TNFα levels remained

similar at 6 weeks and 3 months post-opt compared to pre-opt (20.6±1.4 pg/mL, n=6 and 22.4±2.6 pg/mL, n=4 vs 21.6±1.1 pg/mL, n=5, respectively).

The effect of time (p=0.0002) but not T2DM status (p=0.374) nor its interaction with time (p=0.699) was statistically significant on the average plasma leptin levels, however, the post-hoc analysis did not detect specific significant differences between the cohorts at any time point. However, the average plasma leptin levels decreased in both cohorts at post-opt compared to pre-opt. In the non-T2DM cohort, the average plasma leptin levels significantly decreased at 6 weeks and 3 months post-opt (35.1±7.5 ng/mL, n=9, p=0.02 and 42.4±5.9 ng/mL, n=5, vs 83.5±9.3 ng/mL, n=14, respectively). Similarly in the T2DM cohort, the average plasma leptin levels significantly decreased at 6 weeks and 3 months post-opt compared to pre-opt (36.9±6.1 ng/mL, n=7, p<0.05 and 27.3±6.4 ng/mL, n=4 vs 75.0±7.1 ng/mL, n=6, respectively).

The effect of time (p=0.015) but not T2DM status (p=0.919) nor its interaction with time (p=0.531) was statistically significant on the average plasma adiponectin levels, however, the post-hoc analysis did not detect specific significant differences between the cohorts at any time point. However, the average plasma adiponectin levels increased in both cohorts at post-opt compared to pre-opt. In the non-T2DM cohort, the average plasma adiponectin levels increased at 6 weeks and 3 months post-opt compared to pre-opt, however, the difference observed was not statistically significant (8.6±1.2 µg/mL, n=9 and 8.1±1.3 µg/mL, n=4 vs 6.1±0.7 µg/mL, n=12, respectively). Similarly in the T2DM cohort, the average plasma adiponectin levels increased at 6 weeks compared to pre-opt, however, the difference observed was not statistically significant (8.4±1.3 µg/mL, n=5 and 9.7±1.0 µg/mL, n=4 vs 4.7±1.0 µg/mL, n=6, respectively).

6.5.3.2.2 Plasma biomarkers of insulin sensitivity

The effect of time (p=0.783), T2DM status (p=0.097) nor their interaction (p=0.622) was significantly significant on the average fasting plasma glucose levels, therefore, no significant differences were observed between the two cohorts at any timepoint. The average fasting plasma glucose levels remained similar in both cohorts at post-opt compared to pre-opt. In the non-T2DM cohort, the average fasting plasma glucose levels were not significantly altered at 6 weeks and 3 months post-opt compared to pre-opt (3.8±0.2 mmol/L, n=6 and 3.9±0.2 mmol/L, n=4 vs 3.6±0.3 mmol/L, n=13, respectively). Similarly in the T2DM cohort, the average fasting plasma glucose levels were not significantly altered at 6 weeks and 3 months post-opt compared to pre-opt (4.2±0.7 mmol/L, n=3 and 4.5±0.7 mmol/L, n=3 vs 4.9±0.8 mmol/L, n=6, respectively).

The effect of time (p=0.011) (but not T2DM status (p=0.859)) and its interaction with T2DM status (p=0.002) were statistically significant on the average fasting plasma insulin levels, however, the post-hoc analysis did not detect specific significant differences between the cohorts at any time point. The average fasting plasma insulin levels decreased in both cohorts at post-opt compared to pre-opt. In the non-T2DM cohort, the average fasting plasma insulin significantly levels decreased at 6 weeks and 3 months post-opt compared to pre-opt (17.0±2.5 µIU/mL, n=6, p=0.038 and 18.0±3.2 µIU/mL, n=4 vs 23.9±1.9 µIU/L, n=13, respectively. Similarly in the T2DM cohort, the average fasting plasma insulin levels decreased 6 weeks and at 3 months post-opt compared to pre-opt, however, the difference observed was not statistically significant (18.7±2.5 µIU/mL, n=4 and 10.3±2.2 µIU/mL, n=3 vs 29.7±4.5 µIU/mL, n=6, p=0.022, respectively).

The effect of time (p=0.027) (but not T2DM status (p=0.925)) and its interaction with T2DM status (p=0.004) were statistically significant on the average HOMA-IR, however, the post-hoc analysis did not detect specific significant differences between the cohorts at any time point. The average HOMA-IR was reduced in both cohorts at post-opt compared to pre-opt. In the non-T2DM cohort, the average HOMA-IR reduced at 6 weeks and 3 months post-opt compared to pre-opt, however, the difference observed was not statistically significant (2.0±0.3, n=6 and 2.1±0.4, n=4 vs 2.9±0.3, n=9, respectively). Similarly in the T2DM cohort, the average HOMA-IR decreased at 6 weeks and 3 months post-opt compared to pre-opt, however, the difference observed was not statistically significant (2.5±0.3, n=4 and 1.3±0.2, n=3 vs 3.6±0.5, n=6, respectively).

The effect of time (p=0.013) (but not T2DM status (p=0.399)) and its interaction with T2DM status (p=0.026) were statistically significant on the average HOMA-%S, however, the post-hoc analysis did not detect specific significant differences between the cohorts at any time point. The average HOMA-%S was improved in both cohorts at post-opt compared to pre-opt. In the non-T2DM cohort, the average HOMA-%S improved at 6 weeks and 3 months post-opt compared to pre-opt, however, the difference observed was not statistically significant (57.9±12.0 %, n=6 and 52.0±10.5 %, n=4 vs 36.2±3.1, n=9, respectively). Similarly in the T2DM cohort, the average HOMA-%S was improved at 3 months post opt compared to pre-opt and 6 weeks post opt, however, the difference observed was not statistically significant (41.8±5.6 %, n=3 and 93.4±25.2 %, 30.7±4.1 %, n=6, respectively).

The effect of time (p=0.198), T2DM status (p=0.420) nor their interaction (p=0.305) was statistically significant on the average HOMA-%β, therefore, no significant differences were observed between the two cohorts at any timepoint. The average HOMA-%β

remained similar in both cohorts at post-opt compared to pre-opt. In the non-T2DM cohort, the average HOMA-%β remained similar at 6 weeks and 3 months post-opt compared to pre-opt (264.3±12.4 %, n=6 and 268.1±33.0 %, n=4 vs 296.4±24.6 %, n=9, respectively). Whereas, in the T2DM cohort, the average HOMA-%β reduced at 6 weeks and 3 months post-opt compared to pre-opt, however, the difference observed was not statistically significant (252.6±23.0 %, n=3 and 167.4±55.0 %, n=3 vs 314.7±70.6 %, n=6, respectively).

6.5.3.2.3 Plasma gut hormone

The effect of time (p=0.001) (but not T2DM status (p=0.569) nor its interaction with time (p=0.834)) was statistically significant on the average plasma GLP-1 levels, however, the post-hoc analysis did not detect specific significant differences between the cohorts at any time point. The average plasma GLP-1 levels increased in both cohorts at post-opt compared to pre-opt. In the non-T2DM cohort, the average plasma GLP-1 levels increased at 6 weeks post-opt (313.7±15.3 ng/mL vs 257.7±14.8 ng/mL, n=13, respectively) and significantly at 3 months post-opt compared to pre-opt (339.2±25.7 ng/mL, n=4 vs 257.7±14.8 ng/mL, n=13, p=0.029, respectively). Similarly, in the T2DM cohort, the average plasma GLP-1 levels increased at 6 weeks (306.3±43.7 ng/mL, n=3 vs 278.1±21.1 ng/mL, n=6, respectively) and significantly at 3 months post-opt compared to pre-opt (374.8±21.9 ng/mL, n=3 and vs 278.1±21.1 ng/mL, n=6, p=0.038, respectively).

Plasma Biomarkers: Type 2 diabetes mellitus Status							
		Pre-opt		6 Weeks Post-opt		3 Months Post-opt	
Type 2 diabetes mellitus Status		Non-T2DM	T2DM	Non-T2DM	T2DM	Non-T2DM	T2DM
Inflammatory Biomarkers							
	CRP (µg/mL)	12.1±2.0 (n=12)	10.6±6.3 (n=6)	8.8±2.8 (n=7)	11.3±3.8 (n=7)	5.4±1.9 (n=4)	10.8±7.3 (n=3)
	Annexin A1 (ng/mL)	1.2±0.1 (n=14)	1.1±0.1 (n=6)	1.3±0.1 (n=9)	1.6±0.3 (n=7)	1.6±0.2 (n=5)	1.6±0.2 (n=4)
	IL-1β (pg/mL)	1.2±0.2 (n=12)	1.5±0.5 (n=5)	1.3±0.2 (n=9)	1.5±0.3 (n=4)	2.3±0.4 (n=5)	2.1±0.4 (n=4)
	IL-10 (pg/mL)	7.2±1.0 (n=12)	6.4±0.8 (n=6)	10.0±1.2* (n=8)	9.6±1.0 (n=6)	9.3±0.9 (n=4)	8.8±1.4 (n=4)
	TNFα (pg/mL)	21.7±0.4 (n=14)	21.6±1.1 (n=5)	21.5±1.1 (n=9)	20.6±1.4 (n=6)	21.1±1.5 (n=5)	22.4±2.6 (n=4)
	Leptin (ng/mL)	83.5±9.3 (n=14)	75.0±7.1 (n=6)	35.1±7.5* (n=9)	36.9±6.1* (n=7)	42.4±5.9 (n=5)	27.3±6.4 (n=4)
	Adiponectin (µg/mL)	6.1±0.7 (n=12)	4.7±1.0 (n=6)	8.6±1.2 (n=9)	8.4±1.3 (n=5)	8.1±1.3 (n=4)	9.7±1.0 (n=4)
Insulin Sensitivity							
	Fasting Glucose (mmol/L)	3.6±0.3 (n=13)	4.9±0.8 (n=6)	3.8±0.2 (n=6)	4.2±0.7 (n=3)	3.9±0.2 (n=4)	4.5±0.7 (n=3)
	Fasting Insulin (µIU/mL)	23.9±1.9 (n=13)	29.7±4.5 (n=6)	17.0±2.5* (n=6)	18.7±2.5 (n=4)	18.0±3.2 (n=4)	10.3±2.2 (n=3)
	HOMA-IR	2.9±0.3 (n=9)	3.6±0.5 (n=6)	2.0±0.3 (n=6)	2.5±0.3 (n=4)	2.1±0.4 (n=4)	1.3±0.2 (n=3)
	HOMA-%S (%)	36.2±3.1 (n=9)	41.8±5.6 (n=3)	57.9±12.0 (n=6)	93.4±25.2 (n=3)	52.0±10.5 (n=4)	30.7±4.1 (n=6)
	HOMA-%β (%)	296.4±24.6 (n=9)	314.7±70.6 (n=6)	264.3±12.4 (n=6)	252.6±23.0 (n=3)	296.4±24.6 (n=9)	167.4±55.0 (n=3)
Gut Hormone							
	GLP-1 (ng/mL)	257.7±14.8 (n=13)	278.1±21.1 (n=6)	313.7±15.3 (n=6)	306.3±43.7 (n=3)	339.2±25.7* (n=4)	374.8±21.9* (n=3)

Table 6.6: Plasma biomarkers analysis of non-T2DM and T2DM cohorts at pre- and post- Bariatric surgery. *Plasma inflammatory biomarkers, fasting glucose and plasma insulin levels and plasma gut hormone were analysed and HOMA-IR, HOMA-%S and HOMA-%β were calculated using the equations presented in section 6.4.2.2. One-way ANOVA followed by Tukey's Post-hoc multiple comparison test was used to statistically analyse the data. Data presented as Mean±SEM.*
*P value was set at p <0.05 and is denoted by *vs pre opt*
Pre-opt= Pre-operatively, Post-opt= Post-operatively, T2DM= Type 2 diabetes mellitus, CRP= C-reactive protein, IL-= Interleukin, TNFα= Tumour necrosis factor α, HOMA-IR= Homeostatic model assessment of insulin resistance, HOMA-%S= Homeostatic model assessment of insulin sensitivity, HOMA-%β= Homeostatic model assessment of pancreatic β cell function and GLP-1= Glucagon like peptide 1

6.5.3.3 Annexin A1

The effect of time (p=0.080), T2DM status (p=0.679) nor their interaction (p=0.505) was statistically significant on the average plasma ANXA1 levels, therefore, no significant differences were observed between the two cohorts at any timepoint. However, the average plasma ANXA1 increased both cohorts at post-opt compared to pre-opt. In the non-T2DM cohort, the average plasma ANXA1 levels increased at 6 weeks and 3 months post-opt compared to pre-opt, however, the difference observed was not statistically significant (1.3±0.1 ng/mL, n=9 and 1.6±0.2 ng/mL, n=5 vs 1.2±0.1 ng/mL, n=14, respectively), as shown in Figure 6.19. Similarly in the T2DM cohort, the average plasma ANXA1 levels increased at 6 weeks and 3 months post-opt compared to pre-opt, however, the difference observed was not statistically significant (1.6±0.3 ng/mL, n=7 and 1.6±0.2 ng/mL, n=4 vs 1.1±0.1 ng/mL, n=6, respectively), as shown in Figure 6.19.

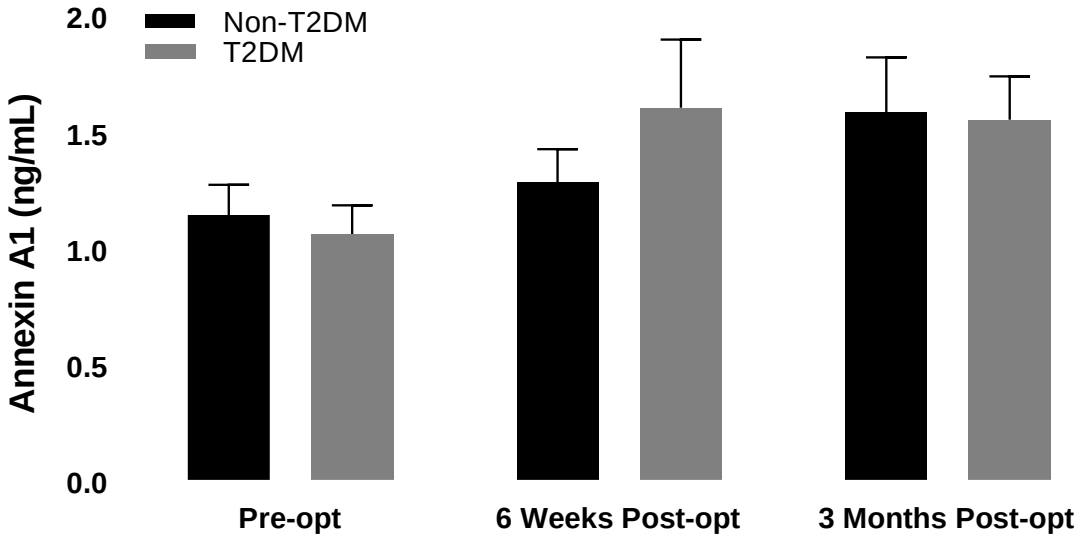


Figure 6.19: Average plasma Annexin A1 (ng/mL) levels of non-T2DM and T2DM cohort pre- and post-Bariatric surgery. *The average plasma Annexin A1 levels were increased at 6 weeks and 3 months post-opt compared to pre-opt in the non-T2DM and T2DM cohorts. However, no statistically significant differences were observed between the cohorts at any time point. Two-way ANOVA followed by Tukey's Post-hoc multiple comparison test was used to statistically analyse the data. Data presented as Mean±SEM. P value was set at p <0.05.*
T2DM= Type 2 diabetes mellitus, Pre-opt= Pre-operatively, Post-opt= Post-operatively

6.5.3.3.1 Annexin A1: Non-Type 2 diabetes mellitus

Plasma ANXA1 levels directly correlated with plasma HDL levels (r=0.323, p=0.058, n=25) (Figure 6.20), significantly inversely correlated with plasma LDL levels (r=-0.340, p=0.048, n=25) (Figure 6.21) and directly correlated with HOMA-%β (r=0.343, p=0.074, n=19) (Figure 6.22) in the non-T2DM cohort.

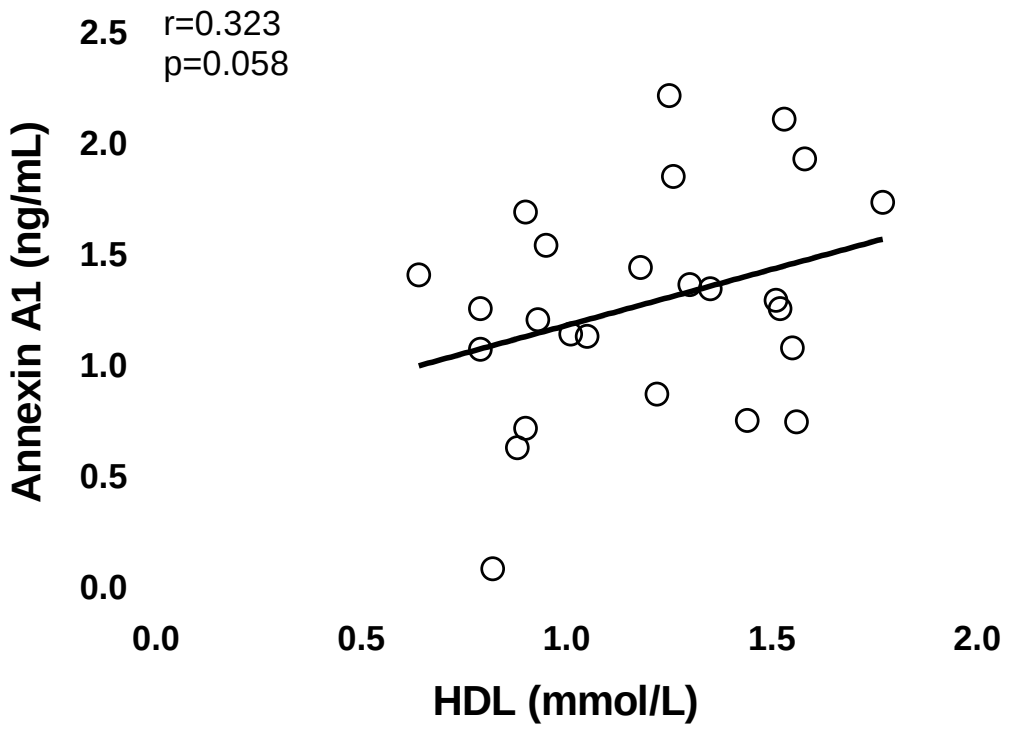


Figure 6.20: Correlation between plasma Annexin A1 levels and plasma HDL in non-T2DM participant cohort. *Plasma Annexin A1 levels directly correlated with plasma HDL levels. One tailed Person correlation coefficient was used to statistically analyse the data.*
HDL= High density lipoprotein

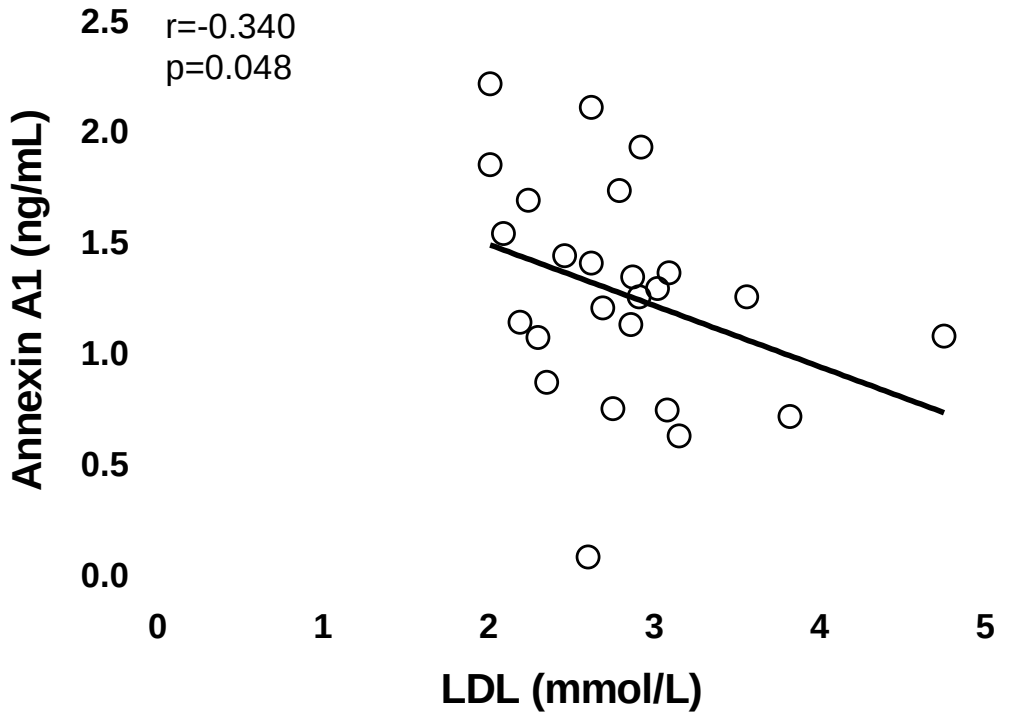


Figure 6.21: Correlation between plasma Annexin A1 levels and plasma LDL in non-T2DM participant cohort. *Plasma Annexin A1 levels significantly inversely correlated with plasma LDL levels. One tailed Person correlation coefficient was used to statistically analyse the data.*
LDL= Low density lipoprotein

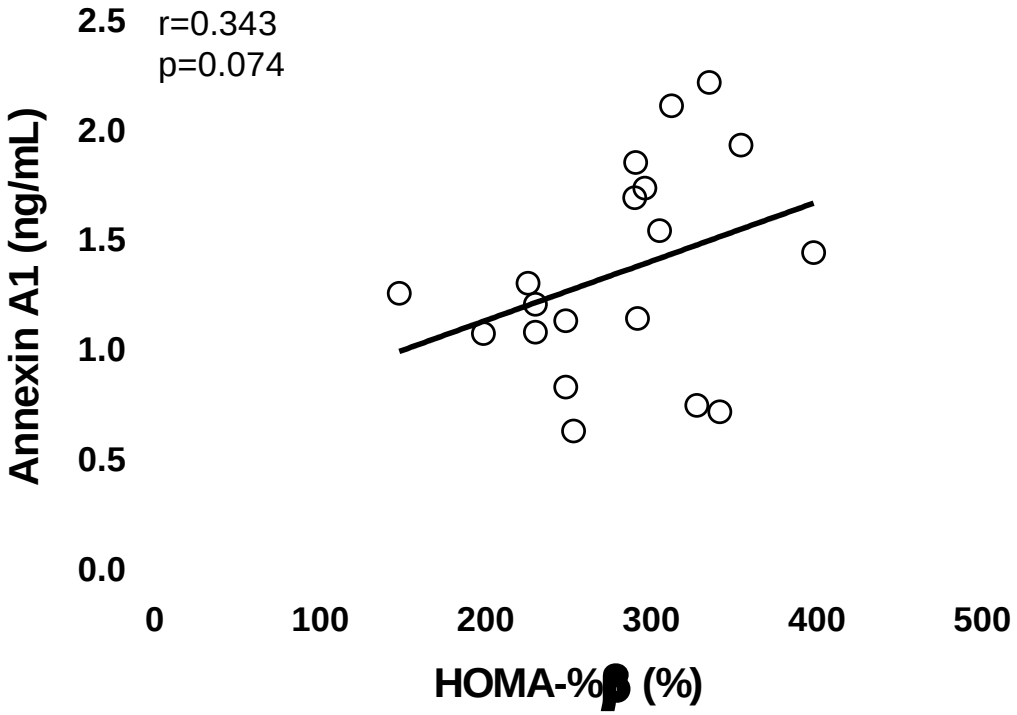


Figure 6.22: Correlation between plasma Annexin A1 levels and HOMA-%β in non-T2DM participant cohort. *Plasma Annexin A1 levels directly correlated with HOMA-%β. One tailed Person correlation coefficient was used to statistically analyse the data.*
HOMA-%β= Homeostatic model assessment of pancreatic β cell function

6.5.3.3.2 Annexin A1: Type 2 diabetes mellitus

Plasma ANXA1 levels inversely correlated with BMI (r=-0.377, p=0.075, n=16) (Figure 6.23), directly correlated with plasma TNFα levels (r=0.419, p=0.053, n=16) (Figure 6.24) and directly correlated with HOMA-%S (r=0.429, p=0.082, n=12) (Figure 6.25) in the T2DM cohort.

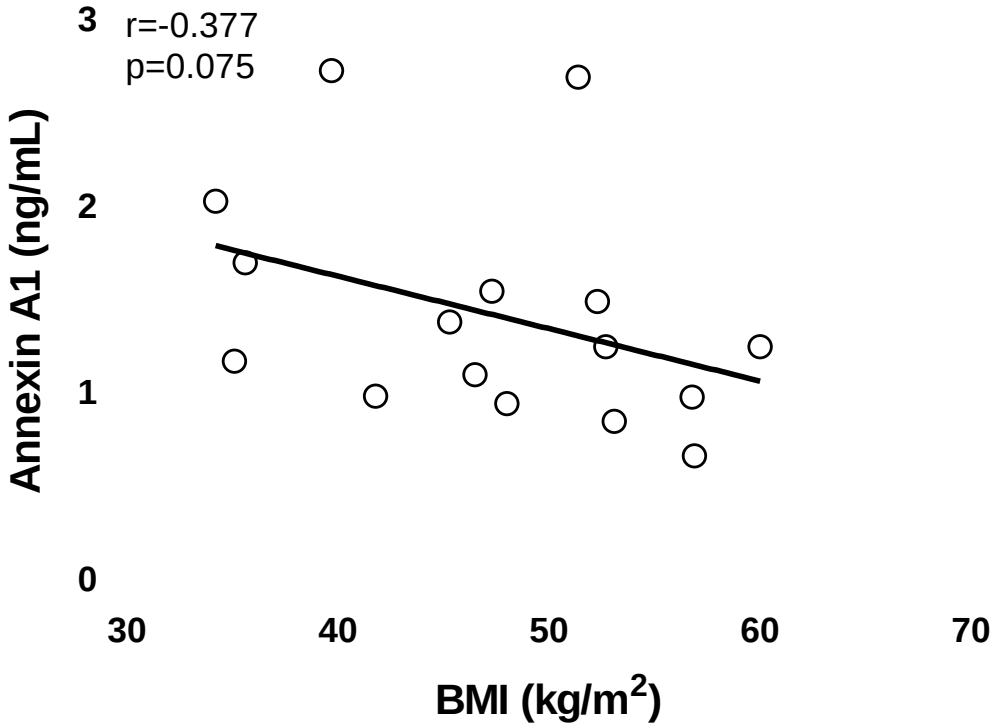


Figure 6.23: Correlation between plasma Annexin A1 levels and BMI in T2DM participant cohort.
Plasma Annexin A1 levels significantly inversely correlated with BMI. One tailed Person correlation coefficient was used to statistically analyse the data.
BMI= Body mass index

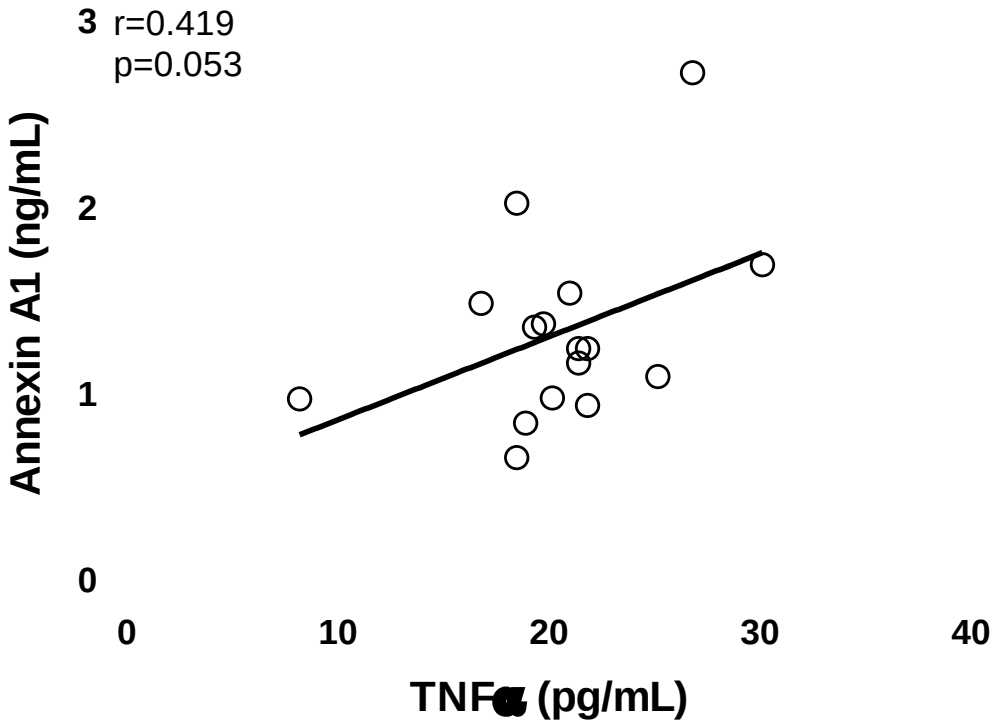


Figure 6.24: Correlation between plasma Annexin A1 levels and plasma TNF α in T2DM participant cohort. *Plasma Annexin A1 levels directly correlated with plasma TNFα levels. One tailed Person correlation coefficient was used to statistically analyse the data.*
TNFα= Tumour necrosis factor α

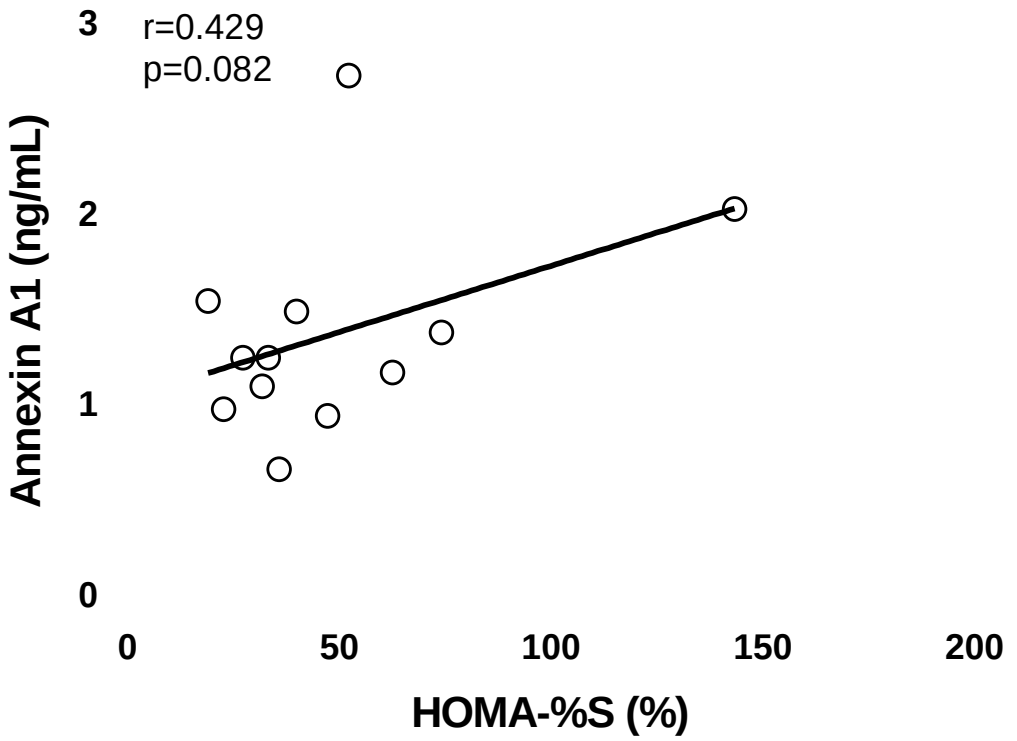


Figure 6.25: Correlation between plasma Annexin A1 levels and HOMA-%S in T2DM participant cohort.
Plasma Annexin A1 levels directly correlated with HOMA-%S. One tailed Person correlation coefficient was used to statistically analyse the data.
HOMA-%S= Homeostatic model assessment of insulin sensitivity

6.6 Discussion

In the current study, the plasma ANXA1 levels were analysed in bariatric surgery patients and correlated with plasma inflammatory biomarkers, markers of insulin sensitivity and gut hormones. Interestingly, plasma ANXA1 levels increased at 6 weeks and 3 months post-opt compared to pre-opt in all studied cohorts, however, a statistical power could not be reached, possibly owing to small participant number as the retention rate throughout the study was relatively poor. Interestingly, no significant difference was observed between the average BMI of SG and RYGB cohort, yet the average plasma ANXA1 levels were higher in the SG cohort at 6 weeks and significantly inversely correlation with BMI in the SG cohort only, perhaps reflecting the less invasive nature of SG and inducing a quicker anti-inflammatory/pro-resolving response compared to RYGB (Frikke-Schmidt, O'Rourke et al. 2016). Interestingly, Pietrani 2018 report higher circulating levels of ANXA1 in obese T2DM patients compared to lean or overweight T2DM patients and a direct correlation with BMI. Whereas, in the current study, the average plasma ANXA1 levels increased in the T2DM cohort at 6 weeks post- compared to pre-opt and significantly inversely correlated with BMI. Furthermore, plasma ANXA1 levels exhibited strong correlations with BMI, plasma lipid levels, plasma CRP, IL-10, IL-1β and TNFα levels, HOMA-%S, HOMA-%β and plasma GLP-1 levels. The correlations between plasma ANXA1 and anthropometric measures, plasma lipids and plasma biomarkers analysed differ between surgery type and T2DM status, suggesting ANXA1 may have different effects in different populations, i.e. non-T2DM vs T2DM.

Approximately, 60-70% of obese individuals have abnormal lipid profiles (dyslipidaemia), including increased circulating LDL levels, increased circulating triglycerides and decreased circulating HDL, thereby, significantly increasing the risk of

cardiovascular diseases in these individuals (Bays, Toth et al. 2013). Bariatric surgery induced weight loss is allied with reduced circulating LDL levels, reduced triglycerides levels and increased circulating HDL levels, thereby significantly reversing dyslipidaemia (Gloy, Briel et al. 2013, Dash, Xiao et al. 2016, Heffron, Parikh et al. 2016).

In the current study, the remission of dyslipidaemia was greater in RYGB cohort compared to the SG cohort and this observation is supported by previous literature (Heffron, Parikh et al. 2016). Furthermore, the results of the current study are the first to show a direct correlation between plasma ANXA1 and plasma HDL levels and inverse correlation between plasma ANXA1 and LDL levels in bariatric surgery patients. Both ANXA1 and HDL mediate cardioprotective effects due to their anti-oxidative, anti-inflammatory and anti-atherogenic properties (Pan, Kong et al. 2016). Increased expression levels of ANXA1were observed in atherosclerotic plaques in carotid stenosis patients without clinical symptoms compared to those with significant cerebrovascular symptoms, suggesting ANXA1 may be involved in resolving and/or attenuating the progression of plaque formation (Cheuk, Cheng 2011). Furthermore, deletion of FPR2/ALX or ANXA1 in Apolipoprotein E (*APO*-/-) high fat diet (HFD) fed mice enhances atherosclerotic lesion formation, adhesion, recruitment and accumulation of arterial myeloid cells, whereas, early atherogenesis and plaque formation is reduced upon treatment with AC2-26 (Drechsler, de Jong et al. 2015). However, FPR2/ALX expression levels are upregulated in human carotid atherosclerotic lesions compared to healthy vessels and is suggested to promote disease progression and increase plaque stability (Petri, Laguna-Fernandez et al. 2015). A recent study reported a novel anti-inflammatory mechanism of HDL through modulating the expression of ANXA1 in vascular

endothelial cells. HDL induced the expression of ANXA1 in endothelial cells in a dose dependent manner and this increase was associated with inhibition of cell surface adhesion molecules and secretion of chemotactic factors (vascular cell adhesion molecule 1, intracellular adhesion molecule 1, E-selection and monocyte chemoattractant protein 1), and inhibiting monocyte adhesion, thereby attenuating formation of atherosclerotic lesions (Pan, Kong et al. 2016). Interestingly, low HDL levels are associated with increased risk of developing T2DM, however, in the current study no significant differences in the plasma HDL levels between T2DM and non-T2DM were observed (Chahil, Ginsberg 2006). Interestingly, the anti-oxidative, anti-inflammatory and anti-atherogenic properties of HDL are suggested to be compromised in diabetes mellitus patients, due to oxidative modification and glycation of HDL proteins (Femlak, Gluba-BrzÃ³zka et al. 2017). In the current study, plasma HDL levels directly correlated with plasma ANXA1 in the non-T2DM cohort but not in the T2DM cohort, possibly reflecting its compromised function in T2DM patients.

Obesity associated inflammation is initiated within the adipose tissue and liver, with elevated macrophage infiltration and expression of proinflammatory cytokines and adipokines. These pro-inflammatory cytokines and adipokines enter the circulation and cause systemic inflammation and contribute to the onset of obesity associated co-morbidities such as, CVD and T2DM (Wood, de Heredia et al. 2009). Changes in cytokine and adipokine synthesis and secretion have been suggested to improve metabolic risk factors in obesity in response to weight loss induced by lifestyle changes and surgical procedures, however, the results reported are inconsistent. In the current study, plasma levels of pro-inflammatory cytokines showed an inconsistent trend suggesting that the circulatory levels all pro-inflammatory cytokines are not regulated in a similar manner

and the invasive nature of surgery may contribute to the discrepancies (Rakotoarivelo, Lacraz et al. 2018).

In the current study, plasma CRP levels were reduced, whereas, plasma IL-1β were increased post-surgery in all studied cohorts. CRP is an acute phase protein involved in regulating the innate immune system and is used as a marker to identify inflammatory diseases as it exacerbates the inflammatory status and induces systemic inflammation (Wang, Nakayama 2010). Circulating levels of CRP are higher in overweight and obese individuals compared to healthy individuals and are associated with distribution of body fat independent of BMI (Visser, Bouter et al. 1999). Elevated CRP levels are an independent risk factor for CVD and insulin resistance and have been suggested to reflect the amount and activity of other pro-inflammatory, pro-atherogenic cytokines such as, TNFα, interleukin 1 (IL-1) and interleukin-6 (Lagrand, Visser et al. 1999, Yudkin, Stehouwer et al. 1999, Tchernof, Nolan et al. 2002). Therefore, the decrease in plasma CRP following bariatric surgery reflects an improved inflammatory status and reduced risk of developing co-morbidities (Rojano-Rodriguez, Valenzuela-Salazar et al. 2014, Netto, Bettini et al. 2015). IL-1β is a pleiotropic cytokine and is involved in regulating pro-inflammatory processes that facilitate monocyte infiltration and migration to the site of inflammation, insulin secretion and appetite regulation (Sica, Wang et al. 1990, Kupper, Groves 1995, Mandrup-Poulsen 1996, Plata-Salaman 1994). Though IL-1β is considered as a pro-inflammatory cytokine, its involvement in regulating gastric acid secretion and emptying, adipocyte development and adipose biology may induce an increase post bariatric surgery (Delikat, Harris et al. 1993, El-Omar 2001). It stimulates gastrin release from enteroendocrine G cells in the gastric antrum and duodenum (Weigert, Schaffer et al. 1996). Gastrin is released in response to food and gastric

distension, acts to increase the secretion of hydrochloric acid, pepsinogen and pancreatic juices and reduces appetite (Meek, Lewis et al. 2016). Circulating levels of gastrin are reduced 12 months post RYGB, whereas, SG has been associated with increased plasma gastrin levels in both human and animal studies, but the exact mechanism that induces this change is unclear (Sundbom, Holdstock et al. 2007, Sillakivi, Suumann et al. 2013, Grong, Arbo et al. 2015). In the current study, plasma IL-1β levels increase in both RYGB and SG cohorts, therefore, the increase in IL-1β may act to increase gastrin levels, reduce appetite and aid in weight loss. The previous literature reports conflicting results regarding the effect of bariatric surgery induced weight loss on circulating TNFα levels (Hafida, Mirshahi et al. 2016). Some authors report no change in circulating TNFα levels following RYGB, whereas, others report reduced plasma TNFα following SG and RYGB (Catalan, Gomez-Ambrosi et al. 2007, Sams, Blackledge et al. 2016, Viana, Araujo-Dasilio et al. 2013, Netto, Bettini et al. 2015). In the current study, no significant changes in plasma TNFα levels were observed post- compared to pre-opt. Furthermore, in the current study, direct correlations between plasma ANXA1 levels and CRP, TNFα and IL-1β were observed, whereas, an inverse correlation between plasma ANXA1 and CRP in obesity has been reported (Kosicka, Cunliffe et al. 2013), suggesting the direct correlation may act as a compensatory mechanism underlying systemic inflammation and possibly masking the effect of ANXA1. Furthermore, the direct correlation with plasma IL-1β may suggest a role for ANXA1 in regulating appetite, adipose tissue development and body weight.

IL-10 is a pleiotropic anti-inflammatory cytokine involved in immunoregulation and inflammation. It is synthesised and secreted by macrophages, dendritic cells, B cells, $CD4^+$, $CD8^+$ and Th2 T cells and inhibits the major histocompatilibty complex class II

and costimulatory molecules on monocytes and macrophages, and limits the synthesis and secretion of pro-inflammatory cytokines and chemokines in T-helper 1 T cells and natural killer cells by inhibiting nuclear factor kappa β to aid in resolving inflammation (Couper, Blount et al. 2008). IL-10 is considered to play a protective role in human metabolism, as it is shown to provide protection against endothelial dysfunction (by inhibiting superoxide thereby, supressing impaired endothelium-dependent relaxation) during diabetes mellitus (Gunnett, Heistad et al. 2002). Plasma IL-10 levels are significantly increased after weight loss induced by lifestyle changes and bariatric surgery, and significantly correlates with plasma adiponectin levels (Jung, Park et al. 2008, Netto, Bettini et al. 2015, Wolf, Wolf et al. 2004). Adiponectin induces monocytes and macrophages towards an anti-inflammatory phenotype by inducing synthesis and secretion of IL-10 in human obesity, thus, the increase in plasma adiponectin levels after bariatric surgery may act as a stimulant for increasing plasma IL-10 levels in these individuals (Wolf, Wolf et al. 2004, Jung, Park et al. 2008). Furthermore, recombinant ANXA1 stimulate IL-10 secretion by macrophages primed with liposaccharide in a dose dependent manner, suggesting ANXA1 may partly mediate its anti-inflammatory actions by modulating the secretion of IL-10 (Ferlazzo, D'Agostino et al. 2003). However, plasma IL-10 and plasma ANXA1 levels increased post- compared to pre-opt in the current study, no correlation was observed, suggesting the increase in plasma IL-10 may be independent of plasma ANXA1. Plasma adiponectin and leptin levels are significantly altered following bariatric surgery and reflect the decrease in adipose tissue mass, increased insulin sensitivity and reduced inflammation. In the current study, plasma adiponectin levels directly correlated with plasma ANXA1 levels reflecting the insulin sensitising role of ANXA1. Furthermore, direct correlation with adiponectin suggests, plasma ANXA1

levels may be regulated by total adiposity. The endocrine function of ANXA1 may be compromised in obesity and may primarily act in an autocrine, paracrine manner in the adipose tissue to resolve inflammation, however, this effect may be reversed in response to weight loss, therefore, aiding in reducing systemic inflammation and the risk of developing obesity associated co-morbidities.

Insulin sensitivity and T2DM status are improved following bariatric surgery independent of weight loss (Batterham, Cummings 2016). This improvement is associated with changes in the hormonal secretion in the gut that have a positive influence on insulin secretion, sensitivity and glucose tolerance (Fried 2013). RYGB is considered to produce a greater reduction in insulin resistance soon after the surgery, possibly due to increased secretion of incretins that enhance insulin sensitivity, however, SG, despite being based on a different technique also improves insulin sensitivity to a similar magnitude (Benaiges, Flores Le-Roux et al. 2013). In the current study, HOMA-IR, HOMA-%S and HOMA-%β in the fasted state were used to assess insulin resistance, insulin sensitivity and insulin secretion from pancreatic β cells, respectively. As expected, in the current study, HOMA-IR decreased and HOMA-%S increased, suggestive of decreased insulin resistance and improved insulin sensitivity post bariatric surgery in all study cohorts (Benaiges, Flores Le-Roux et al. 2013, Ferrannini, Mingrone 2009). Furthermore, a HOMA-%β value close to 100% reflects healthy pancreatic β cell function and a decrease in HOMA-%β index indicates improved pancreatic β function post-opt. Previous literature reports increased circulating levels of ANXA1 in obese T2DM patients compared to healthy weight and overweight T2DM patients as well as non-T2DM controls and no correlation with BMI (Pietrani, Ferreira et al. 2018, Purvis, Collino et al. 2019). However, in the current study, no difference in plasma ANXA1 between obese

T2DM and non-T2DM cohort was observed pre-operatively. Furthermore, plasma ANXA1 levels increased following surgery in T2DM and non-T2DM participants in a similar manner and presented an inverse correlation with BMI in the T2DM cohort only. Evidence suggests ANXA1 may be important for regulating blood glucose levels in a diseased state (Purvis, Solito et al. 2019). ANXA1-/- mice HFD fed develop a more severe diabetic phenotype, characterised by increased blood glucose levels, elevated insulin levels and augmentation in oral glucose tolerance test (Purvis, Collino et al. 2019). Furthermore, ANXA1 is suggested to be a key regulator of RhoA activity which restores insulin receptor substrate 1 signalling and recombinant human ANXA1 is proposed as a novel candidate for treatment of T2DM (Purvis, Collino et al. 2019). Therefore, the direct correlation between plasma ANXA1 and HOMA-%S in the T2DM observed in the current study may indicate a role of ANXA1 in improving the glycaemic index and T2DM status of obese T2DM patients following bariatric surgery. Furthermore, ANXA1 is reported to stimulate insulin secretion from rat pancreatic and MIN6N8s cells (Ohnishi, Tokuda et al. 1995) and in the current study a direct correlation between plasma ANXA1 levels and HOMA-%β was observed in the non-T2DM cohort. However, HOMA-%β is not considered a suitable indicator for evaluating improvements in insulin secretion due to the changes in hormones with incretin effect post-surgery as these hormones will only influence insulin secretion postprandially, therefore, HOMA-%β may not truly reflect the efficiency of insulin secretion (Benaiges, Flores Le-Roux et al. 2013). Thus, in addition to fasted state, it would be useful to correlate plasma ANXA1 and markers of insulin resistance, sensitivity and secretion postprandially to identify discrepancies.

The rapid metabolic effects of bariatric surgery have been linked to the changes in the synthesis and secretion of pancreatic and gut hormones. Several gut hormones are

involved in regulating appetite, satiety and gastric emptying and motility (Meek, Lewis et al. 2016). PYY and GLP-1 are secreted by the L cells in the gut and reduce gastric emptying and motility thereby, promoting satiety. Reduced gastric emptying is associated with improved glucose tolerance as it results in a more gradual supply of nutrients to the gut (Schirra, Katschinski et al. 1996). The increase in postprandial PYY and GLP-1 levels post bariatric surgery are unclear, however, the 'hindgut' hypothesis suggests this rise is due to anatomical changes or increased intestinal transit (Nausheen, Shah et al. 2013, Strader, Vahl et al. 2005, Yousseif, Emmanuel et al. 2014). The 'foregut' hypothesis suggests the exclusion of the upper small intestine is responsible for the changes, possibly due to the decreased secretion of the anti-incretin factor (Meek, Lewis et al. 2016). In the current study, fasting plasma GLP-1 levels significantly increase post-opt regardless of surgery type and T2DM status and directly correlated with plasma ANXA1 levels. The direct correlation further reflects the insulin sensitising effects of ANXA1, which could be partly mediated by modulating the expression of DPP4.

6.7 Conclusion

Due to the low participant numbers (owing to several factors including, no incentives, pre-opt stress and travelling from afar) and poor retention rate (owing to several factors including, delayed surgeries and participants unable to attend nor reschedule their post-opt appointments in sufficient time to meet the timelines of this study) throughout the study, a firm conclusion cannot be formed, however, plasma ANXA1 levels show increasing trends in relation to decreasing BMI in all studied cohorts. The limitations of this study include, small cohort, only women during the follow up, limited follow up and non-randomisation.

However, the remission of dyslipidaemia and increase in plasma HDL levels (in particular) after bariatric surgery induced weight loss may partly modulate the expression of ANXA1 to reduce the risk of developing obesity associated co-morbidities, thus, it would be of significant interest to study correlations between ANXA1 and markers of cardiovascular risk is well in obese individuals and bariatric surgery patients.

Chapter 7: Evidence of aberrant inflammation in patients with Lipodystrophy

Plasma Annexin A1 levels inversely correlate with adiposity and markers of inflammation in obesity, however, it is unclear, whether this decrease is due to increasing fat mass or presence of low-grade chronic inflammation. Lipodystrophy patients have little to no fat mass yet display similar metabolic abnormalities as obese individuals. Therefore, the objective of this study was to investigate the plasma Annexin A1 levels in lipodystrophy patients and correlate it with anthropometric measures and inflammatory biomarkers to elucidate the relationship between plasma Annexin A1 levels, adiposity and inflammation.

7.1 Abstract

Introduction: Lipodystrophy and obesity represent extreme and opposite ends of the adiposity spectrum. Lipodystrophy diseases are characterised by abnormal accumulation, distribution and/or total loss of the adipose tissue. They are characterised by increased metabolic abnormalities and elevated risk of developing co-morbidities such as, type 2 diabetes mellitus, like obese individuals.

Aim: The aim of this study was to analyse plasma Annexin A1 (ANXA1) levels in lipodystrophy patients and correlate with inflammation to understand the relationship between ANXA1, adiposity and inflammation.

Methodology: Plasma samples of Lipodystrophy patients were gifted by Professor David Savage, Metabolic Research Laboratories, Cambridge University and were compared against body mass index (BMI) matched controls. Plasma samples were analysed, and results were statistically analysed using GraphPad Prism 5.0. Statistical significance at 95% was determined using One-tailed unpaired students T-test and One-tailed Pearson's correlation coefficient.

Results: Plasma ANXA1 levels were significantly reduced in lipodystrophy patients of all genotypes in comparison to control and inversely correlated with BMI and plasma C-reactive protein levels.

Conclusion: Therefore, it is proposed that attenuated levels of ANXA1 may play a role in inducing insulin resistance and development of associated co-morbidities by failing to resolve inflammation in obesity and by failing to regulate insulin sensitivity in lipodystrophy.

7.2 Introduction

Lipodystrophies are a heterogenous group of diseases characterised by generalised or partial loss of adipose tissue. If the loss is localised, it is often associated with pathological accumulation of lipids in non-adipose tissue depots (Mantzoros 2012). Lipodystrophies have been reported in the literature for more than 100 years, but only recently new subsets of diseases have been recognised. The loss of adipose tissue resulting from genetic mutations (autosomal dominant and recessive subtypes) is extremely rare and loss resulting from autoimmune disorders or drugs such as, protease inhibitors or insulin injections is more common (Herranz, de Lucas et al. 2008). The adipose tissue of lipodystrophic patients is characterised by smaller adipocytes possibly due to abnormally low expression of crucial adipogenic transcription factors such as, peroxisome proliferator activated receptor γ (PPARγ) and adipocyte differentiation related genes, greater cell size variation, disruption of cell membranes and signs of apoptosis (Bastard, Caron et al. 2002, Jan, Cervera et al. 2004).

Lipodystrophy diseases are associated with similar metabolic abnormalities as obese individuals, such as, hypertriglyceridemia, dyslipidaemia, hyperinsulinemia and insulin resistance and the onset of co-morbidities such as, type 2 diabetes mellitus (T2DM), hepatic steatosis, cardiovascular disease and cancer (Brown, Araujo-Vilar et al. 2016, Phan, Reue 2005). The severity of the metabolic abnormalities positively correlates with the degree of adipose tissue lost (Haque, Shimomura et al. 2002). For example, complete loss of adipose tissue in congenital generalised lipodystrophies (CGL) and acquired generalised lipodystrophies (AGL) induces extreme insulin resistance, whereas, partial loss of adipose tissue in familial partial lipodystrophies (FPL) and acquired partial

lipodystrophies (APL) induces mild to moderate insulin resistance with little to no metabolic complications (Haque et al. 2002).

CGL are autosomal recessive diseases caused by mutations of specific genes involved in lipid metabolism and adipocyte function, including *AGPAT2* (CGL1), *BSCL2* (CGL2), *CAV1* (CGL3) and *PTRF* (CGL4) (Table 7.1) (Brown et al. 2016). They are characterised by near to total loss of adipose tissue and prominent muscles at birth or shortly after (Agarwal, Simha et al. 2003). Due to the absence of fully functioning adipocytes, dietary and endogenously synthesised lipids are stored aberrantly in metabolically important tissues such as, muscle and liver inducing lipotoxicity and development of metabolic abnormalities (Van Maldergem, Magre et al. 2002). Similarly, AGL typically associated with autoimmune disorders (Table 7.2), are characterised by gradual loss of adipose tissue in individuals born with normal adipose tissue distribution. The loss typically begins in childhood or adolescence and is more frequent in females than in males (Misra, Garg 2003). CGL and AGL patients develop hyperphagia in early childhood, have accelerated growth, advanced bone age, features suggestive of acromegaly (enlarged hands and feet), acanthosis nigricans and early onset of T2DM (Herranz et al. 2008). Female patients have additional clinical features including hirsutism, clitoromegaly, irregular menstrual periods, polycystic ovaries and/or infertility (Garg 2004).

Subtype	Gene	Molecular Basis	Unique clinical features
CGL1	*AGPAT2*	- 1-Acylglycerol-3-Phosphate O-Acyltransferase 2 catalyses the formation of phospholipids and triglycerides in various organs - AGPAT isoform 2 is highly expressed in the adipose tissue	- Metabolic active adipose tissue is lost - Mechanical adipose tissue is preserved - Lytic bone lesions in long bones are frequent
CGL2	*BSCL2*	- Seipin regulates lipid homeostasis and adipocyte differentiation - Lipid lipogenesis and lipid droplet accumulation in non-adipocytes - Promotes adipogenesis in periods of excess energy	- Both metabolic active and mechanical adipose tissue are lost - Cardiomyopathy - Lytic bone lesions in long bones are occasional - Abnormal morphology of sperm that affects fertility
CGL3	*CAV1*	- Galectin-3 binds and transports fatty acids to lipid droplets	- Metabolically active adipose tissue is lost - Mechanical and bone marrow adipose tissue is preserved - Functional enlargement of the oesophagus - Short stature, hypocalcaemia, vitamin D resistance
CGL4	*PTRF*	- Cavin-1 regulates the expression of caveolins 1 and 3	- Metabolically active adipose tissue is lost - Mechanical and bone marrow adipose tissue is preserved - Cardiomyopathy - Sudden death - Congenital pyloric stenosis - Developmental delay - Muscle weakness

Table 7.1: Subsets of congenital generalised lipodystrophies. *Congenital generalised lipodystrophies (CGL) diseases are characterised by general loss of adipose tissue caused by mutations in genes involved in adipocyte function and lipid metabolism. CGL are subdivided into 4 subtypes based on the gene involved and the severity of clinical features and metabolic abnormalities vary between the subsets.*
AGPAT2= 1-Acylglycerol-3-phosphate O-Acyltransferase 2, BSCL2= Seipin, CAV1= Galectin-3 and PTRF= Cavin-1.

(Herranz et al. 2008)

Subtype	Prevalence	Clinical features
Panniculitis associated AGL	~25%	- Initial development of panniculitis, followed by localised fat loss when lesions heal - Ongoing panniculitis later results in generalised loss of subcutaneous adipose tissue
Auto-immune AGL	~25%	- Gradual generalised fat loss associated with auto-immune disease; juvenile dermatomyositis
Idiopathic AGL	~50%	- Gradual loss of subcutaneous adipose tissue with unclear aetiology

Table 7.2: Subtypes of acquired generalised lipodystrophies. *Acquired generalised lipodystrophy (AGL) syndromes are characterised by general loss of adipose tissue caused by inflammatory and autoimmune diseases. (Herranz et al. 2008, Misra, Garg 2003)*

FPL are rare autosomal dominant diseases caused by mutations of specific genes involved in lipid metabolism and adipocyte function including; LMNA (FPL2), PPARγ (FPL3), PLIN1 (FPL4) and AKT2 (AKT2-lined) (Table 7.3). Accumulation and distribution of adipose tissue is typically normal in FPL patients during early childhood, but selective and progressive loss occurs from various parts of the body around late childhood or puberty (Brown et al. 2016). Patients have reduced adipose tissue in the arms, legs, head and the trunk regions, with excessive accumulation in other parts of the body including the neck, face and intra-abdominal regions, giving them a 'Cushingoid appearance' (Herranz et al. 2008). Metabolic abnormalities are more common and severe in these patients and they are at a higher risk of developing co-morbidities during adulthood (Subramanyam, Simha et al. 2010, Vantyghem, Vincent-Desplanques et al. 2008). Women with FPL experience menstrual irregularities have a higher occurrence of developing polycystic ovarian syndrome and experience severe metabolic abnormalities compared to men (Garg 2000).

Subtype	Gene	Molecular Basis	Unique clinical features
FPL 1	*Unknown*		- Loss of adipose tissue is confined to arms and legs - May develop central obesity - Only reported in women
FPL 2	*LMNA*	- Lamin A is associated with adipocyte differentiation, insulin signalling and PPARγ - Mutations in Lamin A and C result in premature cell death of adipocytes	- Increased amount of adipose tissue in the cervico-facial area - Premature aging - Metabolic laminopathies resembling the metabolic syndrome
FPL 3	*PPARG*	- Peroxisome proliferator activated receptor γ is essential for adipocyte differentiation	- Loss of adipose tissue is more prominent in the calves and forearms - Metabolic abnormalities are more prominent
FPL 4	*PLIN1*	- Perilipin is the most abundant protein coating the surface of lipid droplets - Essential for storage triglycerides and release of fatty acids	- Loss of adipose tissue is more prominent in the lower limbs and buttocks - Muscle hypertrophy may be more prominent in the calves
FPL 5	*CIDEC (autosomal recessive)*	- Cell death inducing DFFA like effector C is expressed and plays a role in storage of lipid within the lipid droplets	- Loss of adipose tissue in the arms and legs - Hypertension and severe insulin resistance
FPL 6	*LIPE (autosomal recessive)*	- Hormone sensitive lipase is involved in triacylglycerol degradation and glycerolipid metabolism	- Partial lipodystrophy - Severe insulin resistance - Fatty liver - Acanthosis nigricans
AKT2-linked	*AKT2*	- Protein Kinase B β is thought to play a role in post receptor insulin signalling and may be involved in regulating PPARγ	- Severe lipodystrophy - Impaired insulin activity

Table 7.3: Subsets of familial partial lipodystrophies. *Familial partial lipodystrophy (FPL) diseases are characterised by partial loss of adipose tissue caused by mutations in genes involved in adipocyte function and lipid metabolism. FPL are subdivided into different subtypes based on the gene involved and the severity of clinical features and metabolic abnormalities vary.*
LMNA= Lamin A, PPARG= Peroxisome proliferator activated receptor γ, PLIN1= Perilipin, CIDEC= Cell death inducing DFFA like effector C, LIPE= Hormone sensitive lipase and AKT2= Protein kinase B β (Herranz et al. 2008, Brown et al. 2016)

Lipodystrophies and obesity represent extreme and opposite ends of the adiposity spectrum; however, they induce similar metabolic abnormalities and increase the risk of developing co-morbidities (Herrero, Shapiro et al. 2010). Lipodystrophic mice display increased systemic inflammation with increased expression of proinflammatory cytokines such as, tumour necrosis factor α (TNFα), interleukin 1β and interleukin 6 (IL6) and decreased expression of anti-inflammatory cytokines such as, interleukin 10 (Herrero et al. 2010). Furthermore, mRNA expression of essential adipokines such as, leptin, adiponectin and resistin are downregulated in the lipodystrophic adipose tissue and are consistent with the degree of lipoatrophy and metabolic abnormalities (Herrero et al. 2010). Human immunodeficiency virus (HIV)-associated lipodystrophy patients undergoing highly active antiretroviral therapy display increased systemic proinflammatory cytokines such as, TNFα and IL6 (Johnson 2004). However, data regarding the plasma profiles of inflammatory biomarkers in other forms of human lipodystrophies is limited. Therefore, comparing the underlaying mechanisms of obesity and lipodystrophies may further our understanding of the relationship between adiposity, inflammation and the onset of the metabolic syndrome.

7.2.2 Aim, objectives and Hypothesis

The aim of this study was to analyse plasma ANXA1 levels in lipodystrophy patients and correlate with an inflammatory biomarker. It was hypothesised that, plasma ANXA1 levels are reduced in lipodystrophy patients and inversely correlate with inflammation.

The objectives of this study were:

- To analyse and correlate the plasma ANXA1 levels in lipodystrophy patients and BMI matched controls
- To analyse and correlate the plasma C-reactive protein (CRP) levels in lipodystrophy patients and BMI matched controls
- To correlate plasma ANXA1 levels with plasma CRP levels of lipodystrophy patients and BMI matched control

7.4 Materials and Methods

7.4.1 Participant samples

The lipodystrophy plasma samples and associated anthropometric data were gifted by Professor David Savage, Metabolic Research Laboratories, Cambridge University and were compared against BMI matched controls.

7.4.2 Metabolic measurements

7.4.2.1 Enzyme linked immunosorbent Assays

Plasma inflammatory biomarkers, fasting plasma insulin levels and fasting plasma gut hormones were quantified using enzyme linked immunosorbent assay (ELISA). The assays were based on the sandwich technique, as described in Chapter 3 (section 3.3).

- The R&D systems Human C-Reactive Protein (CRP) Quantikine ELISA kit (#DCRP00) was used to quantify CRP in plasma samples, following the manufacturers protocol. The mean intra-assay precision was determined using coefficient of variation (CV). The mean CV of the CRP assay was 2.76%.
- The Cusabio Human Annexin I (ANXAI) ELISA kit (#CSB-E12155h) was used to quantify Annexin A1 levels in plasma samples, following the manufacturers protocol. The mean %CV of the ANXA1 assay was 6.38%.

7.4.3 Statistical analysis

Data presented in this study is expressed as Mean ± Standard Error of the Mean (SEM). GraphPad Prism 5 was used to statistically analyse the data and produce the graphs. One-tailed, unpaired students T-test was used to compare control and disease subsets individually and one-tailed Pearson's correlation coefficient to statistically analyse the

data and to assess the differences in the data sets, a p value of $p<0.05$ was considered statistically significant.

7.5 Results

7.5.1 Anthropometric measures

Plasma samples from 9 lipodystrophy patients and 31 BMI matched controls were analysed for ANXA1 and CRP in the current study. No statistically significant difference was observed between the average BMI of lipodystrophy patients (23.9± 1.5 kg/m^2, n=9) and matched controls (23.2±0.6 kg/m^2, n=29), p=0.324 (Figure 7.1).

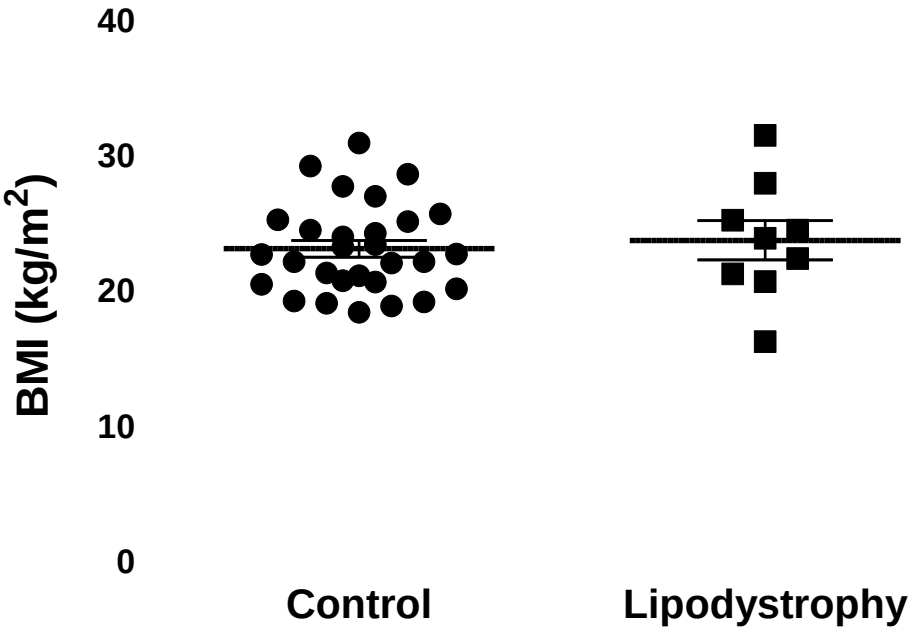


Figure 7.1: Average BMI of lipodystrophy patients and matched controls. *The average BMI of 9 lipodystrophy patients and 29 controls were compared. No statistically significant difference was observed between the two study groups (p=0.324). One-tailed, unpaired student's T-test was used to statistically analyse the data. Data presented as Mean±SEM. BMI= Body Mass Index*

Similarly, no statistically significant difference was observed between the average BMI of lipodystrophy patients and matched controls when characterised per BMI group, underweight (BMI <19.9); 16.4 kg/m^2, n=1 vs 19.1±0.1 kg/m^2, n=1 healthy weight (BMI 20-24.9); 22.7±0.7 kg/m^2, n=5 vs 22.4±0.4 kg/m^2, n=5, p=0.339, overweight (BMI 25-29.9); 26.7±1.4 kg/m^2, n=2, 27.1±0.6 kg/m^2, n=7, p=0.400 and obese (BMI 30+); 31.6 kg/m^2, n=1 vs 31.1±0.0 kg/m^2, n=1, respectively (Figure 7.2).

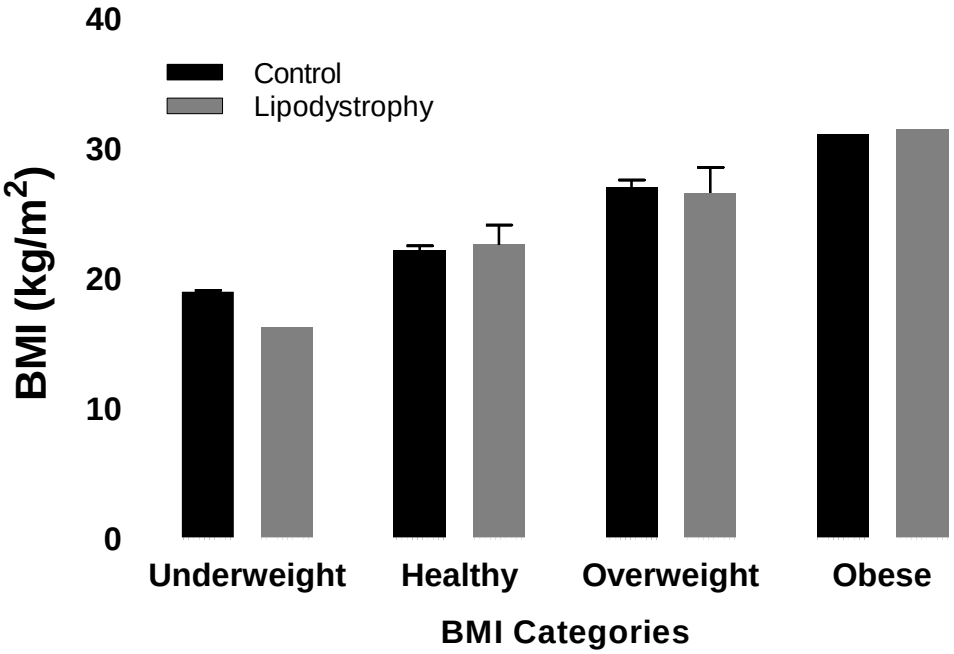


***Figure 7.2: Average BMI of control and lipodystrophy patients per BMI categories**. The average BMI of 9 lipodystrophy patients and 29 controls were compared per BMI category One-tailed, unpaired student's T-test was used to statistically analyse the data. No statistically significant difference was observed between the two study groups who were underweight, healthy (p=0.284), overweight (p=0.400) or obese. Data presented as Mean±SEM. BMI= Body Mass Index*

Furthermore, no statistically significant difference was observed between the average BMI of lipodystrophy patients when characterised per disease subtype; CGL vs FPL (22.0±0.6 kg/m^2, n=2 vs 24.7±1.5 kg/m^2, n=4, p=0.147), CGL vs AGL (20.2±3.8 kg/m^2, n=2, p=0.346) and FPL vs AGL (p=0.118) (Figure 7.3).

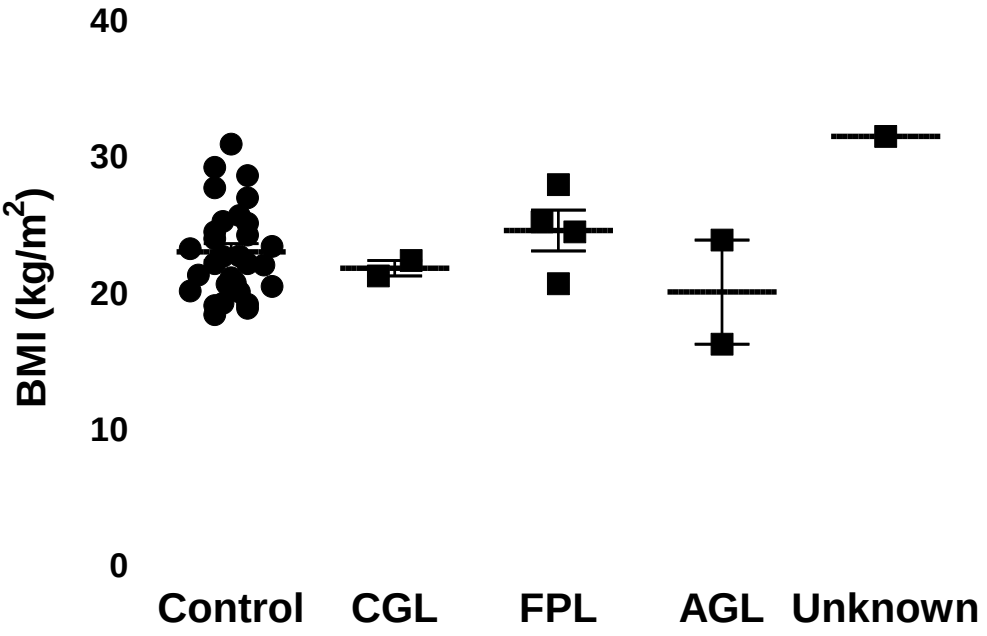


Figure 7.3: Average BMI of lipodystrophy patients based on subtypes. *Lipodystrophy diseases are categorised into different subtypes based on their aetiology and degree of adipose tissue loss. The average BMI of CGL, FPL and AGL were compared. One-tailed, unpaired student's T-test was used to statistically analyse the data. No statistically significant difference was observed between the subtypes; CGL vs FPL (n=2 and n=4 respectively, p=0.147), CGL vs AGL (n=2, p=0.346) and FPL vs AGL (n=4 and n=2 respectively, p=0.118). Data presented as Mean±SEM.*
BMI= Body Mass Index, CGL= Congenital generalised lipodystrophy, FPL= Familial partial lipodystrophy and AGL= Acquired generalised lipodystrophy

7.5.2 Inflammatory Biomarkers

7.5.2.1 Plasma CRP

The average plasma CRP levels were significantly higher in lipodystrophy patients compared to control (3.3±1.0 μg/mL, n=9 vs 1.4±0.3 μg/mL, n=29, p=0.021), as shown in Figure 7.4. However, no significant correlation was observed between plasma CRP levels and BMI, as shown in Figure 7.5. The average plasma CRP levels were higher in healthy weight compared to underweight controls (1.7±0.4 μg/mL, n=5 vs 0.9±0.2 μg/mL, n=17), but was lower in overweight compared to healthy weight controls (1.1±0.5 μg/mL, n=7) and in obese compared to overweight controls (0.47±0.0 μg/mL, n=1), as shown in Figure 7.6. The average CRP levels in lipodystrophy patients followed a similar

pattern when categorised based on BMI. The average CRP levels were higher in healthy weight compared to underweight patients (3.1±1.1 μg/mL, n=5 vs 0.4±0.0 μg/mL, n=1), in overweight compared to healthy weight patients (4.6±4.1 μg/mL, n=2) and lower in obese compared to overweight patients (1.0±0.0 μg/mL, n=1), as shown in Figure 6.6. The average plasma CRP levels were significantly higher in healthy weight lipodystrophy patients compared to control (p=0.034). The average plasma CRP levels were significantly increased in FPL and AGL patients compared to control (3.9±1.8 μg/mL, n=4, p= 0.0079 and 4.0±3.6 μg/mL, n=2, p=0.0254 vs 1.4±0.3 μg/mL, n=29, respectively). However, no significant difference was observed between the average plasma CRP levels between the different lipodystrophy disease subtypes; CGL vs FPL (2.2±1.8 μg/mL, n=2, p=0.283) CGL vs AGL (p=0.336) and FPL vs AGL (p=0.491), as shown in Figure 7.7.

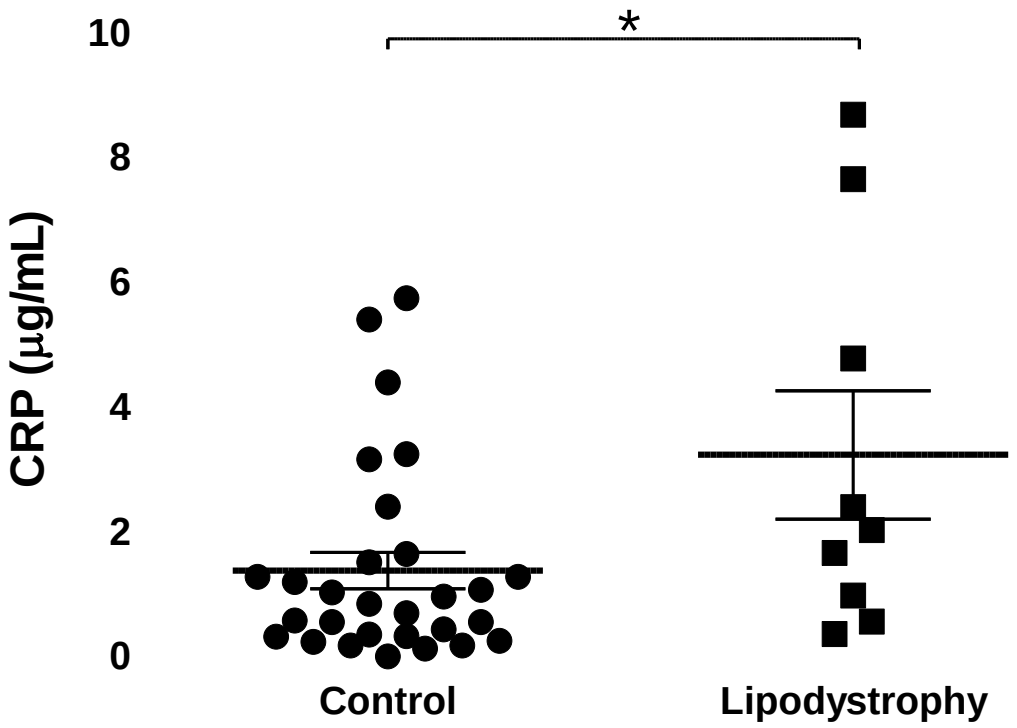


Figure 7.4: Plasma CRP (µg/mL) levels in lipodystrophy patients vs control. *Plasma CRP levels were analysed in 9 lipodystrophy patients and 29 BMI matched controls. One-tailed, unpaired student's T-test was used to statistically analyse the data. The average plasma CRP levels were significantly higher in lipodystrophy patients compared to control (p=0.021). Data presented as Mean±SEM. P value was set at p <0.05 as denoted by *.*
BMI= Body mass index and CRP= C-reactive protein

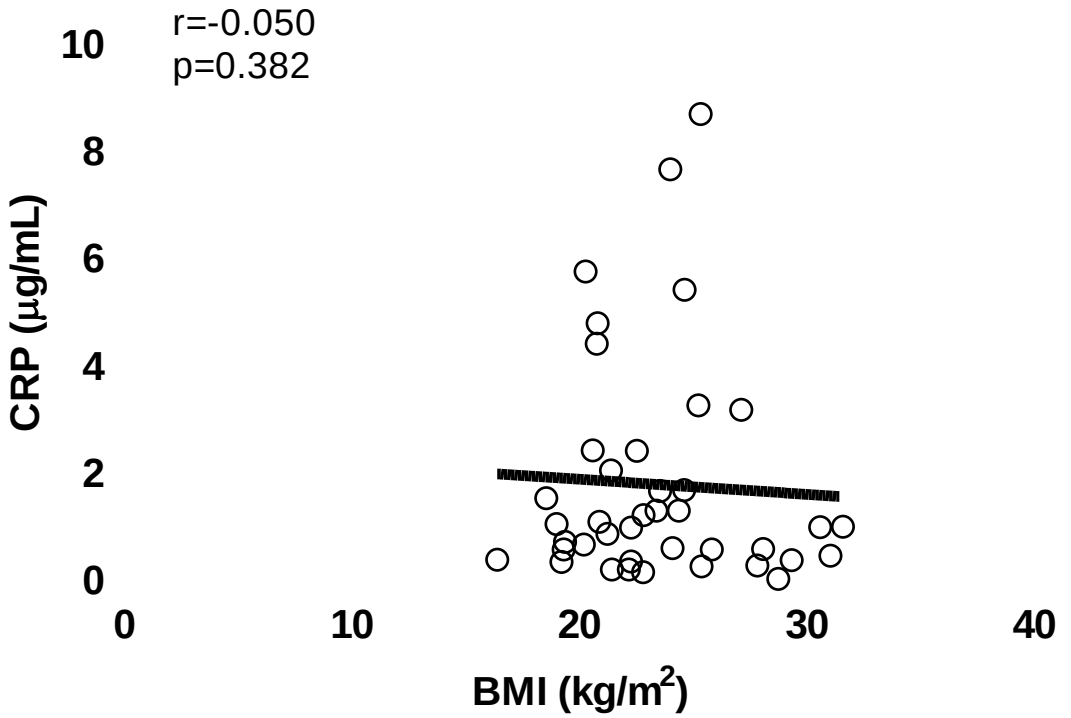


Figure 7.5: Correlation between plasma CRP levels and BMI. *Plasma CRP levels were analysed in 9 lipodystrophy patients and 29 BMI matched controls and correlated with BMI. One tailed Person correlation coefficient was used to statistically analyse the data.*
BMI= Body Mass Index, CRP= C-reactive protein

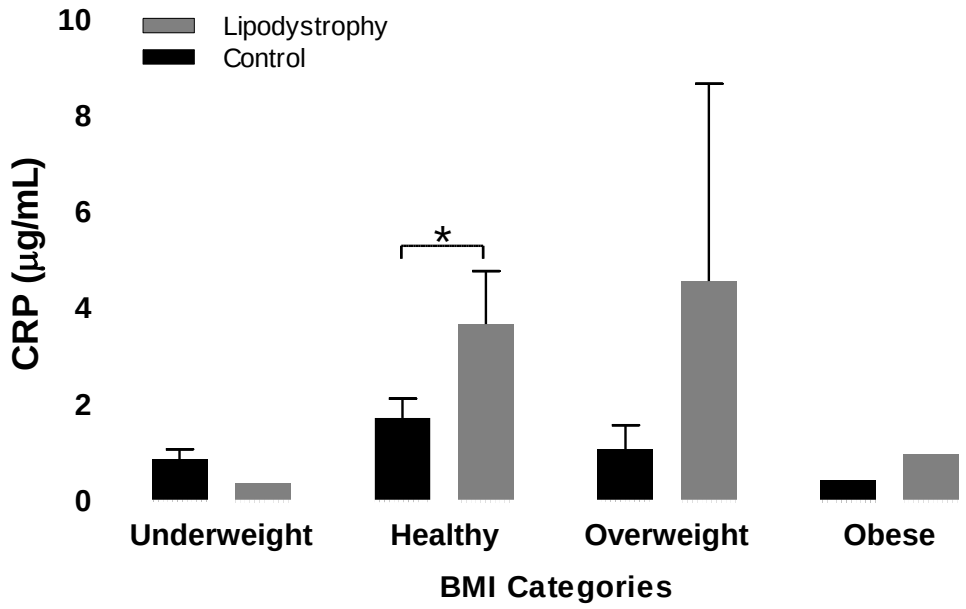


Figure 7.6: Plasma CRP (µg/mL) levels in lipodystrophy patients vs BMI matched control per BMI category. *Plasma CRP levels were analysed in 9 lipodystrophy patients and 29 BMI matched controls. One-tailed, unpaired student's T-test was used to statistically analyse the data. The average plasma CRP levels were higher in healthy weight compared to underweight controls, lower in overweight compared to healthy weight controls and in obese compared to overweight controls, however, no statistically significant difference was observed between each group. Similarly, plasma CRP levels were higher in healthy weight compared to underweight lipodystrophy patients, in overweight compared to healthy weight lipodystrophy patients and lower in obese compared to overweight lipodystrophy patients, however, no statistically significant difference was observed between each group. Plasma CRP levels were significantly higher in healthy weight lipodystrophy patients compared to healthy weight controls (n=5, n=16, p=0.034). Data presented as Mean±SEM. P value was set at p <0.05 and is denoted by *.*
CRP= C-reactive protein, BMI= Body Mass Index

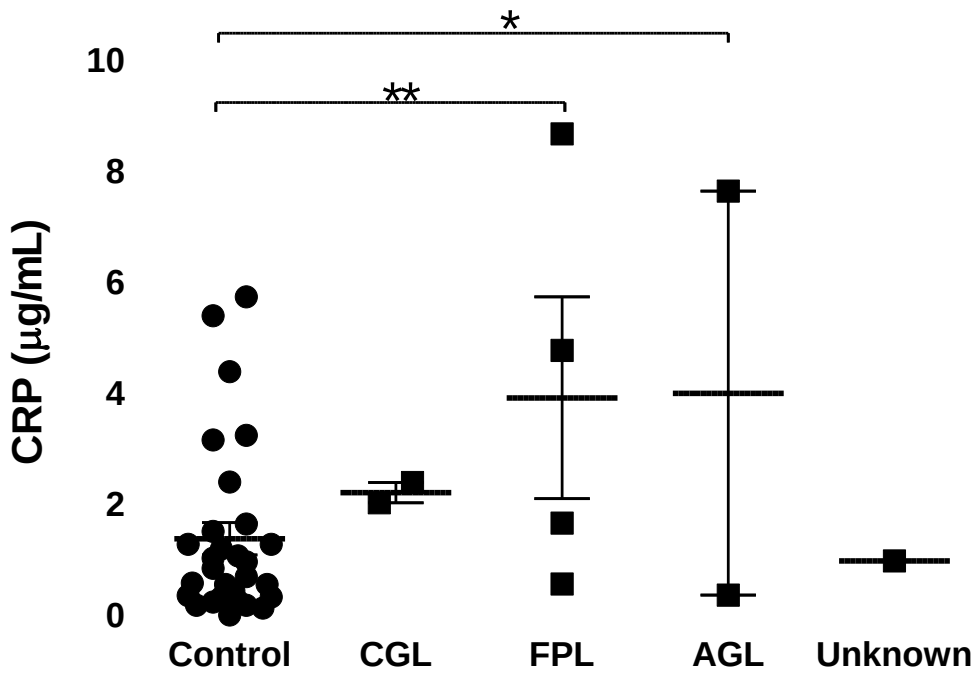


Figure 7.7: Plasma CRP (µg/mL) levels in lipodystrophy patients categorised per disease subtype. *Plasma CRP levels were analysed in 9 lipodystrophy patients and 29 BMI matched controls. One-tailed, unpaired student's T-test was used to statistically analyse the data. Plasma CRP levels significantly higher in FPL and AGL patients compared to controls (n=4, p=0.0079 and n=2, p=0.0283, respectively). No statistically significant difference was observed between the disease subtypes. Data presented as Mean±SEM. P value was set at p <0.05.*
BMI= Body mass index, CGL= Congenital generalised lipodystrophy, FPL= Familial partial lipodystrophy, AGL= Acquired generalised lipodystrophy, CRP= C-reactive protein

7.5.2.2 Plasma Annexin A1

The average plasma ANXA1 levels were significantly lower in lipodystrophy patients compared to controls (0.2±0.1 ng/mL, n=9 vs 0.94±0.1 ng/mL, n=29, p=0.001), as shown in Figure 7.8. Although, an inverse correlation was observed between plasma ANXA1 levels and BMI, it was not statistically significant, as shown in Figure 7.9. The average plasma ANXA1 levels were higher in healthy weight compared to underweight controls (1.3±0.2 ng/mL, n=17 vs 0.7±0.2 ng/mL, n=5), but was lower in overweight compared to healthy weight controls (0.7±0.2 ng/mL, n=7) and in higher in obese compared to

overweight controls (0.019±0.0 ng/mL, n=1), as shown in Figure 7.10. The average ANXA1 levels in lipodystrophy patients followed a similar pattern when categorised based on BMI. The average ANXA1 levels were higher in healthy weight compared to underweight patients (0.2±0.1 ng/mL, n=5 vs 0.01±0.0 ng/mL, n=1), overweight compared to healthy weight patients (0.5±0.03 ng/mL, n=2) and lower in obese compared to overweight patients (0.08±0.0 ng/mL, n=1), as shown in Figure 7.10. The average plasma ANXA1 levels were significantly lower in healthy weight lipodystrophy patients compared to control (p=0.002). The average plasma ANXA1 levels were significantly lower in FPL patients compared to control (0.3±0.1 ng/mL, n=4 vs 0.94±0.1 ng/, n=29, p=0.021). However, no significant difference was observed between the average plasma ANXA1 concentrations between the different lipodystrophy disease subtypes; CGL vs FPL (0.08±0.0 ng/mL, n=2) CGL vs AGL (0.4±0.3 ng/mL, n=4) and FPL vs AGL (p=0.312), as shown in Figure 7.11. Although an inverse correlation between plasma ANXA1 and plasma CRP was observed, it was not statistically significant, as shown in Figure 7.12.

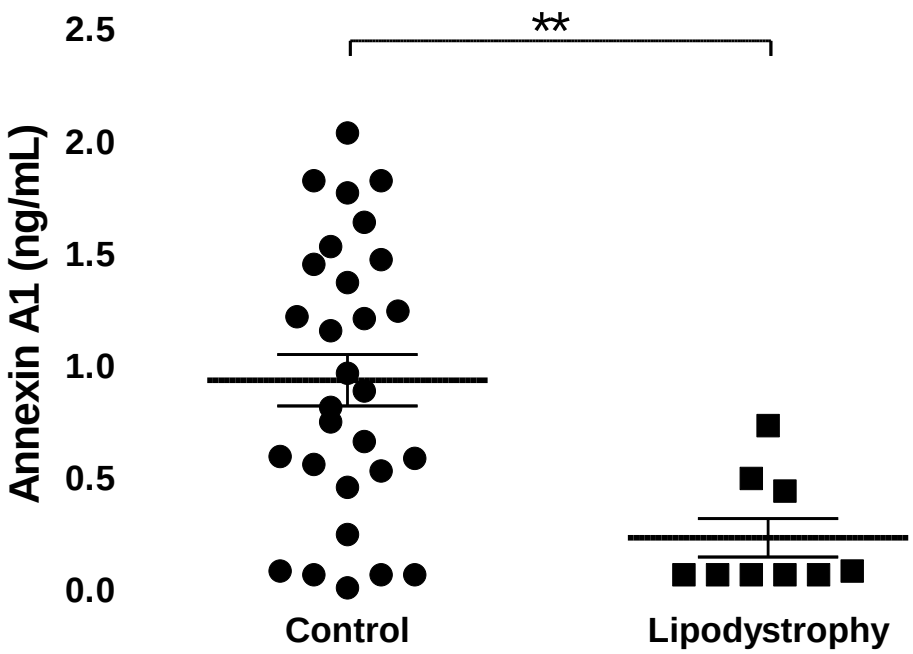


Figure 7.8: Plasma Annexin A1 (ng/mL) levels in lipodystrophy patients vs control. *Plasma ANXA1 levels were analysed in 9 lipodystrophy patients and 29 BMI matched controls. One-tailed, unpaired student's T-test was used to statistically analyse the data. The average plasma ANXA1 levels were significantly lower in lipodystrophy patients compared to controls (p=0.001). Data presented as Mean±SEM. P value was set at p <0.05 and is denoted by *. BMI= Body mass index and ANXA1= Annexin A1*

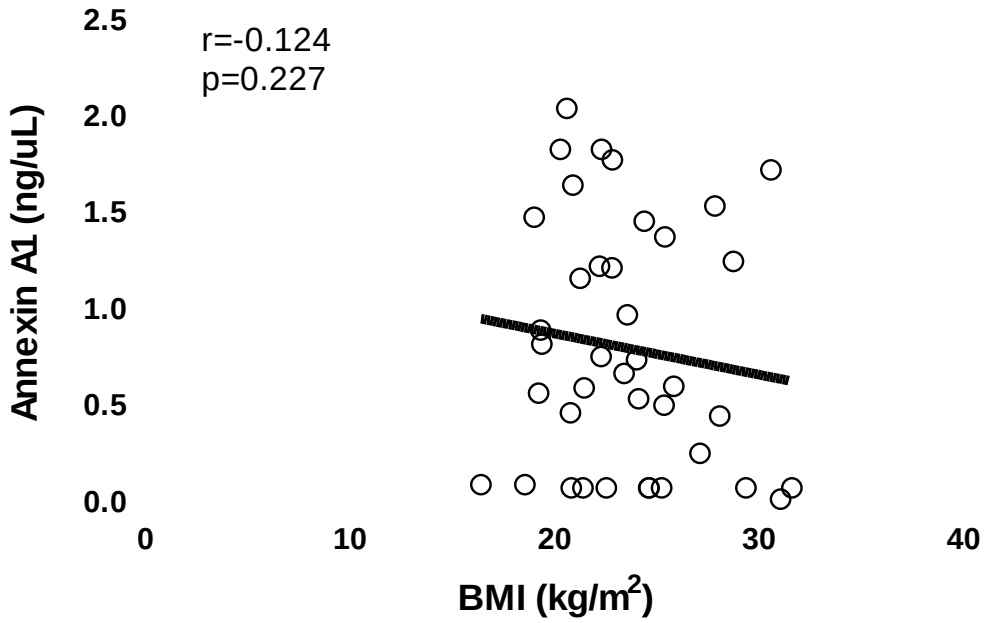


Figure 7.9: Correlation between Plasma Annexin A1 levels and BMI. *Plasma ANXA1 level were analysed in 9 lipodystrophy patients and 29 BMI matched controls and correlated with BMI. One tailed Person correlation coefficient was used to statistically analyse the data. An inverse correlation between the two biomarkers was observed, however it was not of statistical significance. BMI= Body Mass Index and ANXA1= Annexin A1*

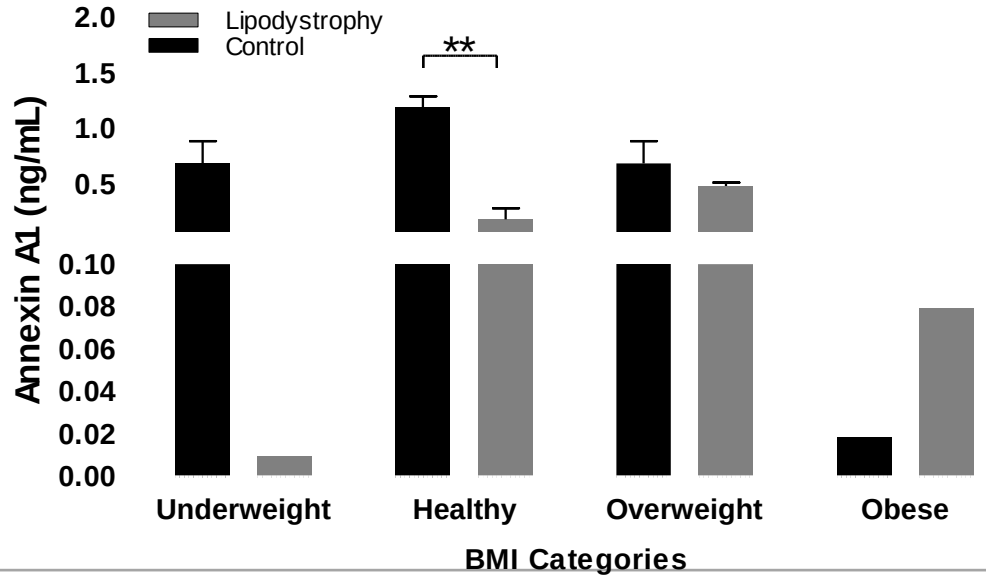


Figure 7.10: Plasma Annexin A1 (ng/mL) levels in lipodystrophy patients vs control per BMI category. *Plasma ANXA1 levels were analysed in 9 lipodystrophy patients and 29 BMI matched controls. One-tailed, unpaired student's T-test was used to statistically analyse the data. The average plasma ANXA1 levels were higher in healthy weight compared to underweight controls, lower in overweight compared to healthy weight controls and in obese compared to overweight controls, however, no statistically significant difference was observed between each group. Similarly, plasma ANXA1 levels were higher in healthy weight compared to underweight lipodystrophy patients and lower in overweight compared to healthy weight lipodystrophy patients and in obese compared to overweight lipodystrophy patients, however, no statistically significant difference was observed between each group. Plasma ANXA1 levels were significantly lower in healthy weight lipodystrophy patients compared to healthy weight controls (n=5 and n=17, respectively, p=0.007). Data presented as Mean±SEM. P value was set at p <0.05 and is denoted by *. BMI= Body Mass Index and ANXA1= Annexin A1*

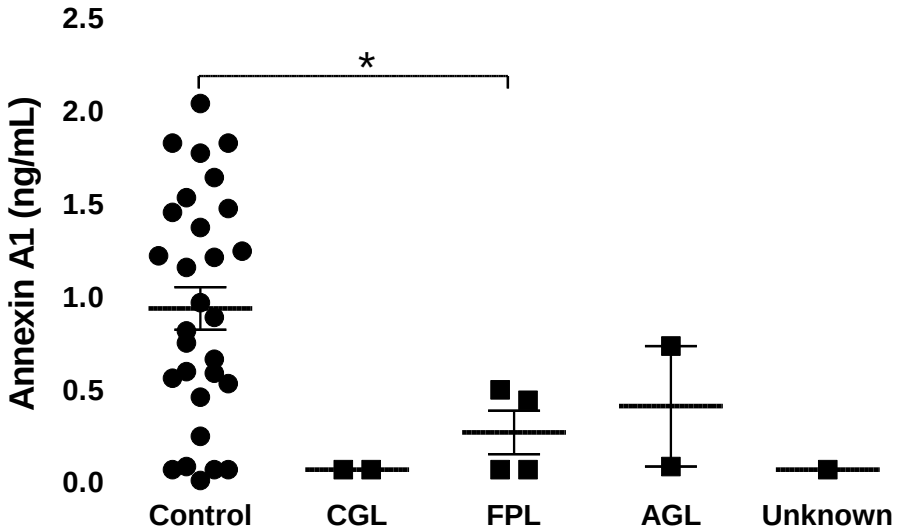


Figure 7.11: Plasma Annexin A1 (ng/mL) levels in lipodystrophy patients characterised per disease subtype. *Plasma ANXA1 levels were analysed in 9 lipodystrophy patients and 29 BMI matched controls. One-tailed, unpaired student's T-test was used to statistically analyse the data. Plasma ANXA1 levels were lower in disease subtypes compared to control. Plasma ANXA1 levels were significantly lower in FPL patients compared to controls (n=4, p=0.021, respectively). No statistically significant difference was observed between the disease subtypes. Data presented as Mean±SEM. P value was set at p <0.05 and is denoted by *. BMI= Body mass index, ANXA1= Annexin A1, CGL= Congenital generalised lipodystrophy, FPL= Familial partial lipodystrophy and AGL= Acquired generalised lipodystrophy.*

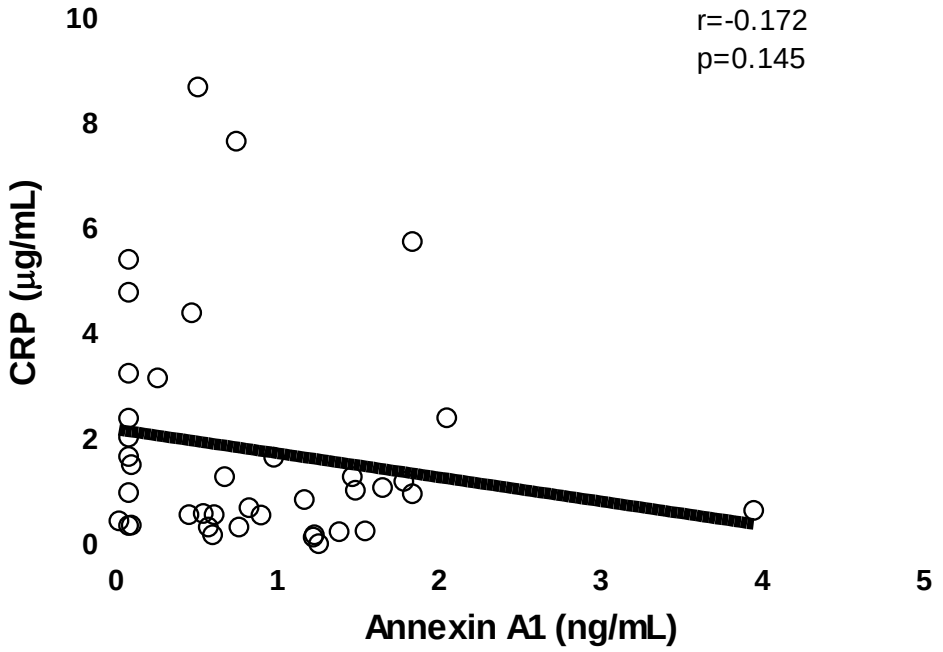


Figure 7.12: Correlation between plasma Annexin A1 and plasma CRP levels. *Plasma Annexin A1 levels and plasma CRP levels were analysed in 9 lipodystrophy patients and 29 BMI matched controls and correlated. An inverse correlation was observed between the two biomarkers however, it was not of statistical significance. One tailed Person correlation coefficient was used to statistically analyse the data. BMI= Body mass index and CRP= C-reactive protein*

7.6 Discussion

In the current study, plasma ANXA1 levels in lipodystrophy patients were analysed and correlated with the pro-inflammatory biomarker, CRP. Interestingly, plasma ANXA1 levels were significantly decreased in lipodystrophy patients compared to control and inversely correlated with BMI, in a similar manner that is observed in obese individuals, suggesting the degree of adiposity may not play a significant role in attenuating plasma ANXA1 levels (Kosicka, Cunliffe et al. 2013). Furthermore, plasma CRP levels were significantly increased in lipodystrophy patients in comparison to controls, suggestive of systemic inflammation and were inversely correlated with plasma ANXA1 levels. The results of the current study indicate plasma ANXA1 levels are attenuated possibly due to an increased inflammatory environment, however, to further understand the relationship between plasma ANXA1 and adiposity, correlation with % body fat (in non-lipodystrophy population) or adipokines may serve as a better indicator than BMI. Furthermore, adiposity and the deposition of fat depots differs amongst lipodystrophy subtypes, however, due to low patient numbers (as low as n=2 for CGL and AGL) the results are inconclusive, therefore, it is of interest to increase subject numbers to investigate relationship between plasma ANXA1 and adiposity.

The origin of inflammation in lipodystrophy varies amongst the different subtypes and may depend on the aetiology. For example, inflammation in congenital lipodystrophies is thought to originate from dystrophic adipose tissue, ectopic lipid accumulation and lipotoxicity (Mantzoros 2012). Whereas in, acquired lipodystrophies induced by autoimmune diseases such as HIV, the increased inflammatory environments created by increased secretion of proinflammatory cytokines and chemokines, and macrophage expression of markers in response to the virus (Ryden, Arner 2007). Anti-retroviral

therapy drugs prescribed to treat HIV further impair adipocyte differentiation by downregulating crucial transcription factors such as PPARγ, induce the generation of reactive oxygen species, increase recruitment of macrophages, inhibit glucose transporter 4 mediated glucose uptake, impair insulin signalling, alter the secretion of adipokines; leptin and adiponectin and enhance production and secretion of proinflammatory cytokines such as IL6 and TNFα (Fiorenza, Chou et al. 2011, Blumer, van Vonderen et al. 2008, Divi, Haverkos et al. 2007, Pacenti, Barzon et al. 2006). Furthermore, they increase lipolysis leading to elevated levels of free fatty acids and ectopic accumulation in non-adipose tissue depots, inducing lipotoxicity and worsening the metabolic profile in these patients (Villarroya, Domingo et al. 2010).

Obesity induced inflammation is hypothesised to be a contributing factor in the development of associated metabolic abnormalities and early on-set of metabolic diseases such as, T2DM however, it is considered to play a differential role in lipodystrophy (Herrero et al. 2010). For example, increased infiltration of classically activated macrophages (M1) into the adipose tissue and increased production of TNFα and IL6 are hypothesized to interfere with the normal activity of insulin receptors and contribute to the development of insulin resistance in obesity (Strissel, Stancheva et al. 2007). Although, macrophage infiltration is also increased lipodystrophic adipose tissue, their phenotype and behaviour (mixture of M1 and M2) is very different. They are believed to be involved in tissue remodelling and repair due to increased apoptosis and not in the pathogenesis of insulin resistance (Herrero et al. 2010). Furthermore, anti-inflammatory drugs known to decrease inflammation and improve hyperglycaemia by lowering glucose and insulin levels in human obese and diabetic individuals do not have similar effects in lipodystrophy mice (Herrero et al. 2010). Instead, the altered synthesis and secretion of

adipokines is hypothesised to induce lipodystrophy associated metabolic abnormalities. Plasma leptin levels positively correlate with adiposity, therefore, it is not surprising that the levels are significantly reduced in CGL and AGL patients, but not as much in FPL patients (Pardini, Victoria et al. 1998, Hegele, Cao et al. 2000). Leptin replacement therapy markedly improves dyslipidaemia, insulin sensitivity, reduces haemoglobin A1c levels and intrahepatic lipid content in lipodystrophy patients (Mantzoros 2012). Similarly, plasma adiponectin levels are significantly reduced in CGL and AGL patients, whereas not so much in FPL and APL patients (Haque et al. 2002). The low levels of adiponectin are proposed to induce insulin resistance and the metabolic syndrome in lipodystrophy (Trujillo, Scherer 2005).

7.7 Conclusion

In addition to its pro-resolving properties, ANXA1 is proposed to mediate insulin sensitising effects, therefore, attenuated levels may contribute to the onset of insulin resistance by failing to resolve inflammation in obesity and by failing to regulate insulin sensitivity in lipodystrophy (Lindgren, Nilsson et al. 2001). Therefore, it would be of interest to correlate plasma ANXA1 levels with glycaemic biomarkers using a larger study cohort (and greater anthropometric measures and biomarkers) to further understand the relationship between attenuated plasma ANXA1 levels and insulin resistance in patients with varying degrees of adiposity.

Chapter 8: General Discussion

The primary aim of this project was to investigate the role of ANXA1 in obesity and associated inflammation. The secondary aims and results are summarised in table 8.1.

Study	Aim	Results
1 (Chapter 4)	To investigate the potential roles of ANXA1 in adipocytes using a model mimicking the inflammatory status that is observed during obesity	Acute AC2-26 treatment significantly downregulated the mRNA expression of pro-inflammatory, insulin resistant genes and upregulated the mRNA expression of anti-inflammatory, insulin sensitive genes.
2 (Chapter 5)	To investigate the role of ANXA1 in the differentiation of preadipocytes into mature adipocytes	ANXA1 may regulate the rate of adipogenesis through differentiational regulation of MAPK intracellular pathways.
3 (Chapter 6)	To investigate the plasma ANXA1 levels in response to rapid weight loss induced by Bariatric Surgery	Plasma ANXA1 levels show increasing trends with decreasing BMI in the full cohort, SG participants, RYGB participants, non-T2DM participants and T2DM participants
4 (Chapter 7)	To investigate the plasma ANXA1 levels in Lipodystrophy patients	Plasma ANXA1 levels are reduced in lipodystrophy patients and inversely correlate with plasma CRP levels

Table 8.1: Summary of Aims and Results of the project.
ANXA1= Annexin A1, MAPK= Mitogen activated protein kinase, BMI= Body mass index, CRP= C-reactive protein, SG= Sleeve gastrectomy, RYGB= Roux-en-Y gastric bypass, T2DM= Type 2 diabetes mellitus

The *in vitro* results from study 1 (Chapter 4) of this thesis indicate several roles of ANXA1 in adipocyte physiology in obesity. The differential regulation of genes unknown to be dysregulated in obesity in response to AC2-26 treatment indicate ANXA1 may be involved in regulating cellular survival and apoptosis, cellular proliferation and differentiation, lipid and glucose metabolism and, intracellular stress and inflammation. It is interesting to note that acute AC2-26 treatment downregulated the expression of genes such as, *CD36, DPP4, LEP* and *TNF* that are involved in inducing insulin resistance and upregulated the expression of genes such as, *ADIPOQ* that are involved in inducing insulin sensitivity in mature obesogenic adipocytes, reflecting an insulin sensitising effect

of ANXA1. Insulin mediates its biological responses by inducing tyrosine phosphorylation of insulin receptor substrate (IRS) proteins which in turn activate downstream signalling pathways (Figure 8.1). Agents such as, inflammatory cytokines, chemokines and adipokines, free fatty acids and cellular stress attenuate insulin activity and induce insulin resistance by modulating IRS phosphorylation (tyrosine phosphorylation activates and serine phosphorylation inhibits the activity) (Gual, Le Marchand-Brustel et al. 2005, Godsland 2009). In addition to changes in phosphorylation of IRS proteins, downregulation of *IRS1* gene expression is considered to play a central role in altered IRS1 tyrosine phosphorylation in adipocytes of obese and T2DM humans and animals (White 2002, Thirone, Huang et al. 2006). Therefore, it was surprising to observe that acute AC2-26 treatment significantly downregulated *IRS1* gene expression in mature obesogenic adipocytes. Though the exact mechanisms and reasoning of this observation are unclear, ANXA1 has shown to promote a dose-dependent inhibition of insulin receptor autophosphorylation, inhibiting its tyrosine kinase activity (Melki, Hullin et al. 1994). These conflicting results warrant further investigation.

Furthermore, acute AC2-26 treatment differentially regulated the expression of genes associated with lipid accumulation and adipogenesis in mature obesogenic adipocytes. It upregulated the expression of genes associated with inhibiting adipogenesis or adipocyte hypertrophy such as, *PPARA* and *PER3,* and downregulated the expression of genes associated with enhancing adipogenesis such as, *PPP3CC,* perhaps indicating a protective role of ANXA1 against adipocyte hypertrophy. Therefore, the role of ANXA1 in adipogenesis was investigated. The *in vitro* results from study 2 (Chapter 5) of this thesis indicate that ANXA1 and its peptide (AC2-26) may regulate the rate of adipogenesis in the latter differential stages through differential regulation of mitogen activated protein

kinase (MAPK) intracellular pathways. Interestingly, mature adipocytes differentiated with specific ANXA1 receptor (FPR2/ALX) antagonist, WRW4 or non-specific receptor agonist, AC2-26 (can activate FPR1 and FPR2/ALX) accumulated significantly less lipids and indicated an immature adipocyte phenotype compared to control. Whereas, mature adipocytes differentiated with low doses of non-specific receptor agonist, BOC-2 (low doses block FPR1 signalling) accumulated significantly more lipids than control. However, no significant difference in lipid accumulation was observed in mature adipocytes differentiated with a high dose of BOC-2 (higher doses block FPR1 and FPR2/ALX). Therefore, the increase in ANXA1 in mature adipocytes and further increase in obese adipocyte suggests, ANXA1 may act in an autocrine and paracrine manner to regulate adipocyte hypertrophy (Kosicka, Cunliffe et al. 2013, Henegar, Tordjman et al. 2008, Alfadda, Benabdelkamel et al. 2013). Hypertrophy is considered to be a key underlying cause of adipocyte dysfunction contributing to the onset of metabolic syndrome and obesity associated co-morbidities, thereby, indicating a protective role of ANXA1, however, this effect may be overshadowed by the sustained presence of nutrients and low grade chronic inflammation (Hirsch, Fried et al. 1989, Danforth 2000, Heilbronn, Smith et al. 2004).

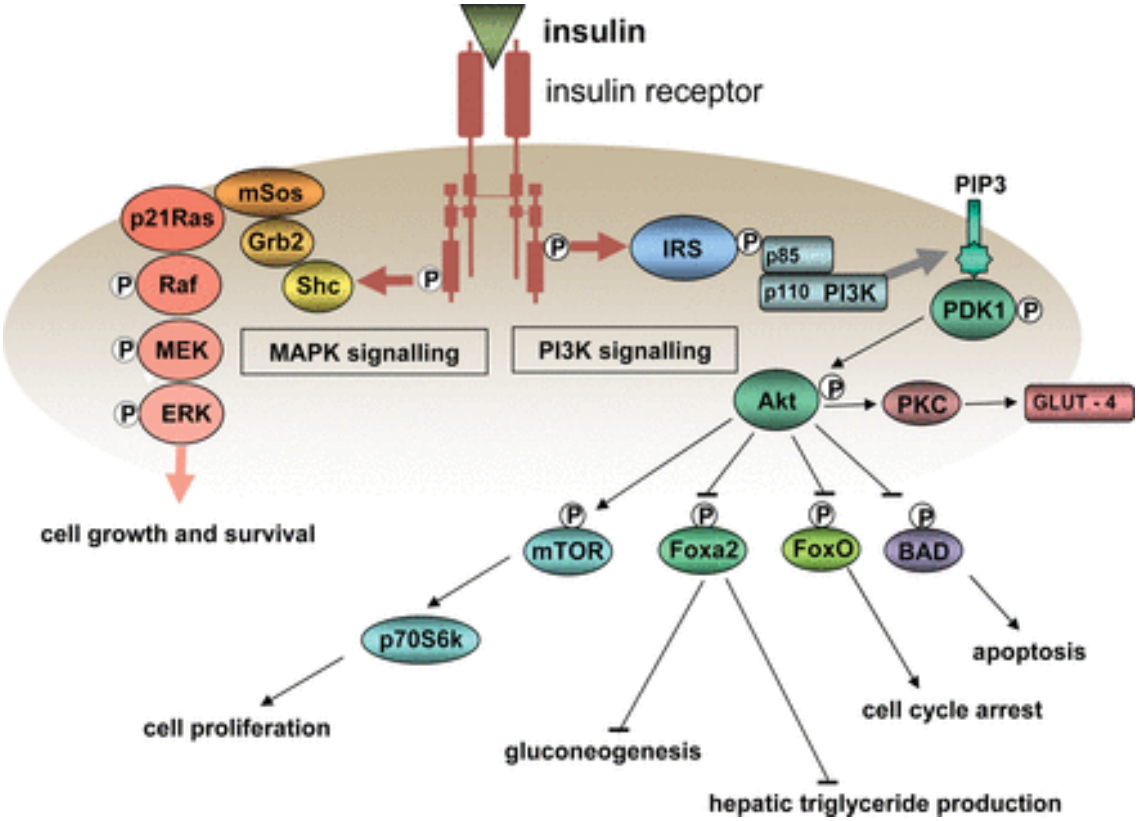


Figure 8.1: Pathways activated by the insulin receptor. *Binding of insulin to the insulin receptor activates two intracellular signalling pathways; MAPK signalling pathway that is involved in cell growth and survival and the PI3K signalling pathway that is involved in cellular proliferation and metabolism.*
MAPK=Mitogen activated protein kinase, PI3K= Phosphoinositide 3 kinase, IRS= insulin receptor substrate

Extracellular ANXA1 is cleaved by proteases to generate smaller peptides including, AC2-26 which are thought to retain the biological functions of ANXA1 (Gavins, Hickey 2012). However, how much ANXA1 is cleaved and how much is remains full length is unknown. Unlike ANXA1, AC2-26 is able to bind to and activate formyl peptide receptor (FPR)-1 and FPR2/ALX (Dalli, Montero-Melendez et al. 2012). The activation of different receptors and intracellular signalling pathways by AC2-26 may contribute to the conflicting results observed in Study 1. Therefore, it would be of interest to investigate the roles of FPR1 and FPR2/ALX individually in mature obesogenic adipocytes and adipogenesis using receptor knockout models. Furthermore, adipocyte differentiation

with FPR1 and/or FPR2/ALX receptor knockouts would further emphasise the results from study 2 (Chapter 5) and confirm the results observed are due to differential activation rather than side-effects of the agonist and antagonists.

The results from the clinical pilot study 3 (Chapter 6) indicate increasing trends of plasma ANXA1 levels with decreasing body mass index (BMI). However, due to small participant numbers and poor retention it is difficult to form a solid conclusion, therefore, a full study in this area is required to determine their association. Interestingly, plasma ANXA1 directly correlated with plasma levels of an incretin hormone, glucagon-like peptide 1 (GLP-1). GLP-1 is proteolytically cleaved by dipeptidyl peptidase 4 (DPP4), thereby inhibiting its incretin effect (Bae 2016). DPP4 inhibitors are a new class of anti-hyperglycaemic agents used to treat T2DM (Ahren 2007). Furthermore, DPP4 is proposed to play a role in linking obesity and the pathogenesis of T2DM by acting as a local mediator of inflammation and insulin resistance in the adipose and hepatic tissues (Deacon 2019). Interestingly, the clinical results from study 1 (Chapter 4) show acute treatment of AC2-26 significantly downregulated the gene expression of *DPP4* in mature obesogenic adipocytes. The increase in plasma ANXA1 in response to weight loss may act in an autocrine, paracrine and endocrine manner to decrease the synthesis and secretion of DPP4, reflected by direct correlations with plasma GLP-1 levels. Therefore, it is of significant interest to further investigate the relationship between ANXA1 and DPP4 locally and systemically to dissect the mechanisms underlaying the development of obesity associated co-morbidities for therapeutic purposes.

Furthermore, the results from the clinical pilot study 3 (Chapter 6) show inconsistent trends. Such as, plasma ANXA1 levels directly correlates with plasma levels of both anti- and pro-inflammatory biomarkers. These reasons behind these associations are unclear,

however, it is speculated that the invasive nature of the surgery may have skewed the findings. Most of the studies carried out in this area report pre- and >12 months post bariatric surgery data; therefore, it is of interest to continue investigating the plasma biomarkers for a longer follow up period. Furthermore, some participants within the study developed post-operative (post-opt) infections, which may have contributed to the inconsistent trends. Thus, the inclusion/exclusion criteria should be reviewed and patients with post-opt infections should be excluded in the future studies, as the biomarkers analysed form part of the inflammatory cascade and post-opt infections could potentially skew the results, giving false positive or negative results.

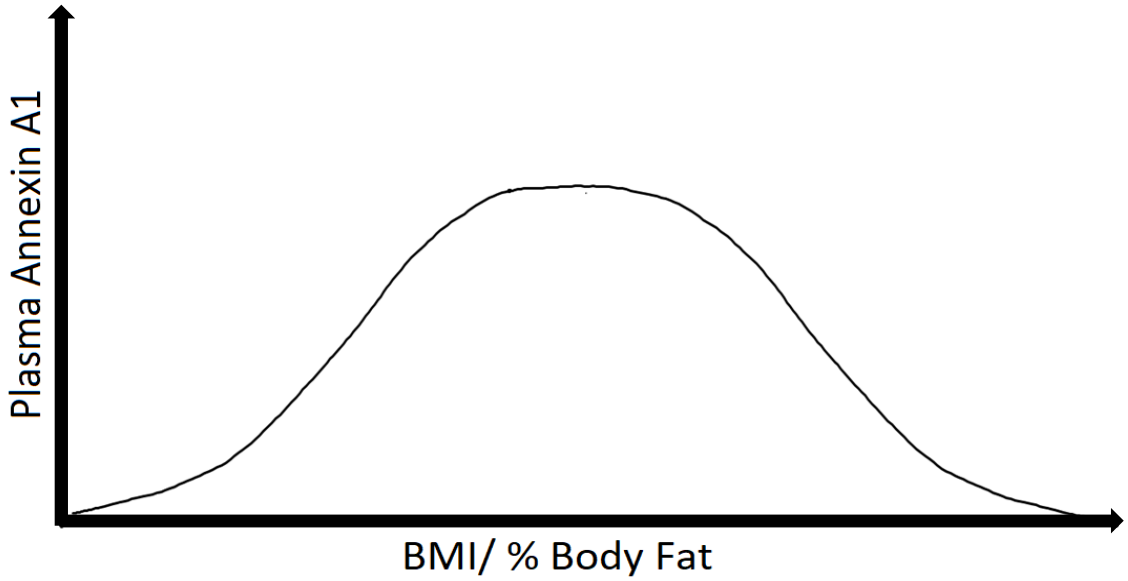


Figure 8.2: Proposed plasma Annexin A1 levels in relation to BMI or % body fat . *Plasma Annexin A1 levels are reduced in individuals with low BMI/%BF and in individuals with high BMI/%Body fat, therefore, it is proposed that plasma Annexin A1 levels follow a normal distribution pattern where the plasma levels are highest in the healthy BMI/%Body fat range.*

Finally, the results from the *in vivo* study 4 (Chapter 7) show plasma ANXA1 levels are significantly reduced in lipodystrophy patients compared to BMI matched controls. This observation is interesting because plasma ANXA1 levels are also significantly reduced in obese individuals and inversely correlate with BMI (Kosicka, Cunliffe et al. 2013) (Figure

8.2). This suggests the regulation of plasma ANXA1 levels may not be in direct relation to the amount of adipose tissue, but rather the presence of local and systemic low-grade chronic inflammation.

8.1 Conclusion

This project describes evidence for two different but overlapping functions of ANXA1. The first of which is produced locally within the adipose tissue depots and may have a role in regulating adipogenesis and reducing adipocyte hypertrophy to prevent adipocyte dysfunction, local inflammation and development of insulin resistance and secondly peripheral circulating levels (the source of which is still unknown) have been demonstrated to correlate with inflammatory biomarkers, markers of insulin sensitivity and gut hormone. Peripherally, ANXA1 may act in an endocrine manner to regulate inflammatory biomarkers to dampen inflammation, regulate insulin secretion and improve the metabolic profile to reduce the risk of developing obesity associated co-morbidities. However, this effect may be overshadowed by the continued increase in systematic inflammation associated with rapid adipose tissue expansion.

8.3 Future Studies

The results of Study 1(Chapter 4) and Study 2 (Chapter 5) indicate a protective role of ANXA1/AC2-26 in mature adipocytes incubated in an environment mimicking the inflammatory status observed in obesity by regulating the expression of genes and pathways associated with key adipocyte functions through alleviating the pro-inflammatory status, restoring metabolic mechanisms and dampening inflammation. To further validate the results of the current studies and to improve the quality and reliability of the data the following studies are suggested:

1) Analyse the expression of genes that were identified to be significantly altered in mature adipocytes that were incubated in an environment mimicking the inflammatory status observed in obesity (Study 1) using gene deletion (CRISPR) or knock down (siRNA) assays. Deleting the ANXA1 gene would further ensure the results are due to the change in ANXA1 rather than a side effect of the agonist treatment, limiting the experimental errors. Furthermore, primary adipocytes obtained from visceral adipose tissue depots could be used as models to investigate if the role or effect of ANXA1/AC2-26 differs amongst different adipose tissue depots.

2) The results of Study 2 indicate that FPR1 is involved in regulating the rate of adipocyte differentiation. Differentiation of SGBS with individual and/or grouped gene deletions or knock downs of ANXA1 and/or its receptors; FPR1 and/or FPR2/ALX into mature adipocytes would further emphasise and validate the role of ANXA1, FPR1 and FPR2/ALX in adipocyte differentiation. Similar experiments would certify the role of ANXA1 and/or its receptors in glucose uptake in mature adipocytes.

3) White adipocyte trans-differentiation to a brown adipocyte is considered a novel therapeutic target for obesity. Acute AC2-26 treatment significantly upregulated the gene expression of *PPARA*, a master regulator of brown adipocyte differentiation in mature obesogenic adipocytes. This data indicates that ANXA1/AC2-26 may induce the expression of genes associated with brown adipocytes in white adipocytes to utilise excess energy in the form of thermogenesis to reduce adipocyte hypertrophy and associated dysfunctions. Therefore, it would be of significant interest to investigate the role of ANXA1/AC2-26 in trans-differentiation for therapeutic purposes.

4) The adipose tissue is a heterogenous tissue therefore, it would be of significant interest to carry out co-culture experiments to investigate the paracrine effects of ANXA1 on immune cells to reflect the physiological state. It would be of significant interest to investigate the effects of ANXA1 synthesised and secreted by adipocytes on monocyte and macrophage migration, and macrophage polarisation.

The question whether plasma ANXA1 is regulated by adiposity or inflammation remains inconclusive. The results of Chapter 5 (Study 3) and Chapter 6 (Study 4) indicate the plasma levels of ANXA1 may be regulated by inflammation, however, it is difficult to form a firm conclusion based on small, short time framed and incomprehensive studies. Therefore, the following developments are suggested:

5) It would be of significant interest to carry out the clinical pilot study 3 on a larger scale (carry out a para-equation based on population size to determine the optimal number of participants required at each follow up) and a longer follow up (on average the studies in this field of study follow up to 12-24 months post opt). Following patients for up to 24 months with follow ups every 3 months would indicate the patterns of plasma ANXA1

proteins and other biomarkers and whether the patters are exponential or reach a plateau. Furthermore, the difference in plasma ANXA1 in response to different bariatric surgeries and diabetes mellitus status are of interest, therefore, a full separate study would be designed to investigate the differences between these groups (wide array of anthropometric measures and plasma biomarkers). If the evidence supports it, ANXA1 agonist could be given to reduce inflammatory effects in patients with T2DM.

6) The clinical pilot study 3 aimed to investigate insulin sensitivity/resistance using oral glucose tolerance test (OGGT) pre-operatively (pre-opt) and post-operatively (post-opt) and correlate it with plasma ANXA1, however, due to small cohort and poor retention, it was not feasible. The OGGT is a more reliable test to determine insulin sensitivity/resistance than using fasting plasma glucose and plasma insulin values. Furthermore, GLP-1 and peptide YY are secreted in response to nutrients, therefore, it would be of significant interest to investigate the plasma levels of these biomarkers pre- and post-opt postprandially than fasted and correlate them with plasma ANXA1 levels to achieve physiologically accurate results.

7) To avoid the effects of surgery, it would be of significant interest to investigate the plasma levels of inflammatory biomarkers in individuals undergoing weight loss through non-surgical methods (lifestyle, exercise and/or diet).

8) In addition to investigating the plasma levels of inflammatory biomarkers and correlating them with ANXA1, it would be of significant interest to investigate the effect of weight loss on the expression and secretion of ANXA1 in adipocytes. However, the exact source of plasma ANXA1 is unknown, therefore it would be of significant interest to investigate what percentage of ANXA1 is secreted by the adipose tissue and how the

percentage changes in response to weight loss, as this would further strengthen the association between ANXA1 and adiposity.

9) It would be of significant interest to investigate the plasma levels of inflammatory biomarkers, insulin sensitivity and adipokines and correlate them with plasma ANXA1 levels in lipodystrophy patients. The results of this study would further contribute to our understanding the regulation of ANXA1 and its relationship with adiposity.

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Appendix A - List of Documents

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Appendix B - Summary of HRA Assessment

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IRAS project ID	220666

Plasma Annexin A1 levels and insulin sensitivity during weight loss through Bariatric Surgery

Abstract

Recently, the adipose tissue has been acknowledged to be more than just a site of lipid storage as it contributes to metabolic homeostasis through the secretion of adipose tissue specific proteins; the "adipokines". Obesity refers to excess lipid storage in the adipose tissue often leading to ectopic deposition in other organs affecting their physiological function. Bariatric surgery; otherwise known as weight loss surgery is thought to be an effective option for individuals who are morbidly obese or have a BMI of over 40. However, individuals often exhibit alterations in the levels of adipokines and insulin sensitivity leading to insulin resistance, despite weight loss. Therefore, the aim of this project is to investigate the levels of an anti-inflammatory protein AnxA1 during weight loss through bariatric surgery. AnxA1 is thought to regulate the function of insulin and preliminary data suggest it decreases the levels of pro-inflammatory markers which may contribute to effective resolution of inflammation and development of diabetes type 2 in these patients.

Introduction

Obesity is a growing pan-endemic health problem in both developing and developed countries. It is a complex disease which results from an increase in fat mass or increased adiposity and is defined as body mass index (BMI) of over 30kg/m^2 or a waist circumference more than 94cm for men and 80cm for women (Goktas, Moustaid-Moussa et al. 2013). It is a chronic disease as it evolves over the years and low grade inflammation causes metabolic changes. Therefore, it is a major risk factor of developing common diseases such as type 2 diabetes, hypertension and cardiovascular disease, thereby significantly increasing total mortality (Lindegaard, Jorgensen et al. 2015). For example; insulin resistance in obesity is thought to be associated to adipose tissue fibrosis, hypoxia and infiltration of immune cells and inflammation leading to cellular hypertrophy, triggering cellular stress processes impairing adipocyte responses to insulin (Moreno-Castellanos, Guzman-Ruiz et al. 2016). Despite recognising the relationship of obesity and the onset of these diseases, the biological link to the pathogenesis is not well understood.

The first recommended treatment for obesity is weight loss through low calorie diets and regular physical exercise. However, lifestyle interventions have low observance and effectiveness in morbidly obese individuals with a BMI of over 40kg/m^2 (Goktas, Moustaid-Moussa et al. 2013). Bariatric surgery is considered to be the best choice for these individuals as it leads to weight loss through restriction and/or malabsorption of nutrients (Adami, Scopinaro et al. 2016). It is an effective and durable treatment in comparison to non-surgical interventions such as life style changes mentioned above and pharmacological therapies. After surgery individuals lose approximately 30% of their body weight and decrease the overall inflammatory response (Goktas, Moustaid-Moussa et al. 2013). In addition to weight loss, bariatric surgery reduces insulin resistance, cardiovascular disease and mortality. However, some patients experience remission of insulin resistance and the onset of diabetes (Adami, Scopinaro et al. 2016). Bariatric surgery causes changes in the adipose tissue biology that impact systemic glucose utilisation thereby affecting insulin sensitivity independent of weight loss (Moreno-Castellanos, Guzman-Ruiz et al. 2016). Metabolic changes observed after bariatric surgery independent of weight loss are thought to result from altered hormonal patterns; i.e upregulation of gastrointestinal insulinotrophic polypeptide (GIP), glucagon-like peptide 1 (GLP-1) and PYY that cause inhibition of appetite, mediating satiety and stimulate insulin production (Goktas, Moustaid-Moussa et al. 2013).

Adipose tissue is a site for storing triglycerides and fats in periods of excess energy intake and releasing them during periods of famine to provide energy. Additionally, it provides mechanical protection for organs and thermal insulation for the organism. It was considered to be a mere site for fat storage but now it is acknowledged as a “metabolically active” endocrine organ. It secretes hormones, cytokines and chemokines, collectively known as ‘adipokines’ that participate in appetite and satiety, insulin secretion and sensitivity, regulating energy homeostasis, inflammation, endothelial function other biological functions(Yang, Lee et al. 2001). Adipokines are detectable both in the plasma and adipose tissue and are active factors that modulate the effect of obesity and related co-morbidities. Dysregulated production of these adipokines in obesity is thought to contribute to the development of chronic low-grade inflammation leading to the onset of associated diseases (Lindegaard, Jorgensen et al. 2015).

Leptin (adipose tissue specific) is a satiety hormone that regulates energy balance by inhibiting hunger, insulin sensitivity and β-cell apoptosis (Goktas, Moustaid-Moussa et al. 2013). The plasma levels of leptin are directly proportional to body fat (Oswal, Yeo 2010). Adiponectin (adipose tissue specific) is involved in regulating glucose levels and fatty acids metabolism in insulin sensitive tissues. It is an anti-inflammatory, anti-apoptotic protein which stimulates energy expenditure and insulin production and sensitivity (Sams, Blackledge et al. 2016). The plasma levels of adiponectin inversely correlate with body fat, insulin resistance and dyslipidaemia. Adiponectin is activated during adipogenesis; however, a feedback inhibition on its production may impose the development of obesity (Yang, Lee et al. 2001). Weight loss through bariatric surgery has shown to have an inverse relationship of plasma adiponectin and weight loss (Sams, Blackledge et al. 2016). As individuals lose weight, there is an increase in plasma adiponectin levels and contributing to the resolution of co-morbidities. In addition, adipose tissue secretes universal cytokines including TNF-α, IL-1β and IL-6. TNF-α is involved in systemic inflammation and is one of the cytokines that make up the in acute phase reaction. The plasma levels of TNF-α directly correlate with body fat and is produced by dysfunctional adipocytes which is thought to impair insulin signalling and secretion(Adami, Scopinaro et al. 2016). IL-1β is a potent pro-inflammatory cytokine that is upregulated in obesity and functionally it induces prostaglandin synthesis, neutrophil influx and T cell activation (Goktas, Moustaid-Moussa et al. 2013). Furthermore, IL-1β secreted from dysfunctional adipocytes is thought to play a role in apoptosis, β cell development and destruction, increasing glycaemic deterioration leading to the onset of diabetes type 2.

The protein of interest in this project is AnxA1; it is a calcium dependent phospholipid binding protein which is produced and secreted by a variety of cell types including adipocytes, neutrophils and macrophages. It exhibits anti-inflammatory properties as it mediates the detachment of neutrophils from the vascular cells through mediating L-selectin shedding, inhibits the activity of phospholipase A2 (inhibiting production of eicosanoids) and inhibits activation of pro-inflammatory CD4+ T cell linages (Rescher, Gerke 2004)(Kosicka, Cunliffe et al. 2013). Plasma AnxA1 levels are decreased in obese in comparison to normal weight individuals showing an inverse relationship (Kosicka, Cunliffe et al. 2013). Interestingly, knockout models have shown opposing results with some reporting increased susceptibility

to weight gain and insulin resistance and others increased weight loss (Akasheh, Pini et al. 2013, Warne, John et al. 2006).

Aims and Hypotheses:

The primary aim of this pilot project is to investigate the changes in AnxA1 and other plasma inflammatory biomarkers, gut hormones and insulin sensitivity in relation to weight loss in individuals undergoing bariatric surgery.

Studies:

1) Changes in body weight of patients over 3 months
2) The effect of weight loss on Plasma AnxA1 levels by types of bariatric procedures
3) The plasma levels of inflammatory biomarkers and their correlation with insulin sensitivity
4) The plasma levels of Gut hormones and their correlation with AnxA1 and inflammatory biomarkers

Hypotheses:

1) Weight loss results in increased plasma AnxA1 levels leading to improved insulin sensitivity leading to effective resolution of inflammation and decreasing the likely hood of developing co-morbidities.

2) Plasma AnxA1 levels directly correlate with anti-inflammatory and inversely correlate with pro-inflammatory markers during weight loss.

3) AnxA1 positively correlates insulin sensitivity and gut hormones

Experimental design and methods

Study population

Participants will be recruited from the bariatric surgical clinic at Heartlands Hospital, Birmingham. Written informed consent will be taken from up to 30 adult Obese participants who meet the criteria for bariatric surgery (Sleeve gastrectomy and Roux-en-Y gastric bypass); Adult subjects Male/Female, aged between 18-75 years, BMI of >40 if do not have type 2 diabetes and >35kg/m^2 if known to have type 2 diabetes. Type 1 Diabetic, have significant

mental health problems or patients receiving long term steroids will be excluded from the study (figure 1).

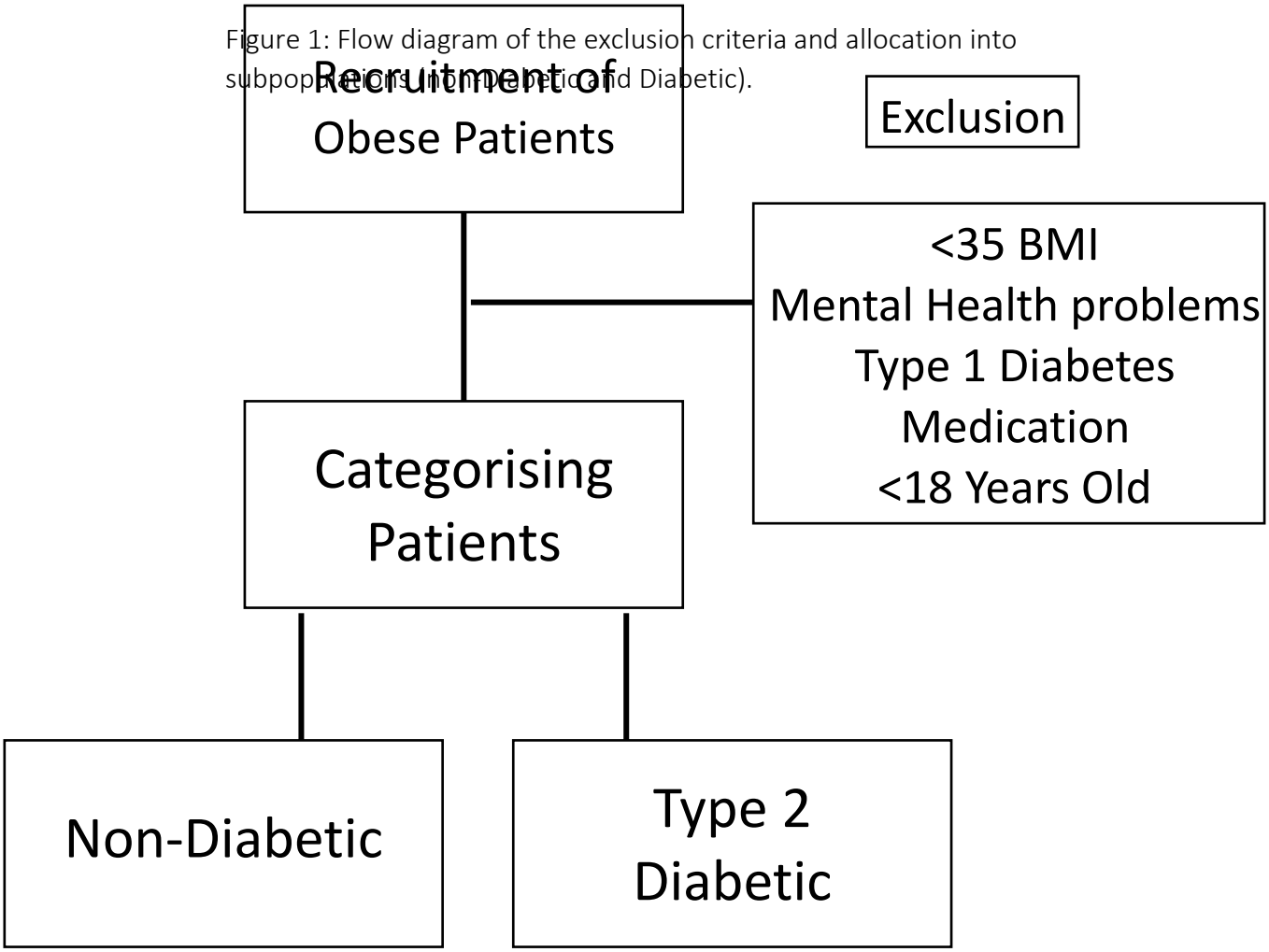

Figure 1: Flow diagram of the exclusion criteria and allocation into subpopulations (Non-Diabetic and Diabetic).

Recruitment

Patients attending the weight management clinics and identified for bariatric surgery will be approached for participation in the study. They will be provided with the patient information sheet, the purpose of the study will be explained and any questions and apprehensions will be discussed. Interested patients will be contacted 48 hours post the initial meeting and if they are willing to take part in the study, they will be invited for a screening visit. After obtaining the informed consent, patients will be screened for eligibility and baseline visit will be agreed.

Study design

Eligible participants will have clinical, biochemical, inflammatory markers and gut hormones measured at baseline and at each of the follow up appointments. A subset of participants who provide informed consent with undergo Oral Glucose Tolerance Test (OGTT) to assess insulin sensitivity. Similarly, the test will be carried out at baseline and at each of the follow up appointments.

Clinical and Biochemical Measures

The following clinical information will be taken from all participants: age, gender, past medical history, treatment, duration of diabetes, weight, height, BMI and systolic and diastolic blood pressure pre-and postoperatively. 5mL of whole blood sample will be collected via venepuncture at the same time and sent to the local routine lab within the hospital for the analysis of biochemical measurements. These include fasting glucose and lipids (total cholesterol, low density lipoprotein-cholesterol (LDL-C), high density lipoprotein-cholesterol (HDL-C) and triglycerides). Insulin and C-peptide will be measured using ELISA as described below. The clinical assessment and collection of blood samples will be done by a trained professional.

Inflammatory markers and Gut hormones

In addition, 10mL of whole blood will be collected in purple top anticoagulant-treated tubes (EDTA-treated) via venepuncture by a trained professional. The samples will be centrifuged for 10 minutes at 1,000-2,000xg to separate into serum and plasma. The supernatant (plasma) will be collected, aliquoted (0.5mL) and stored at -80C.

The plasma will be used to measure range of inflammatory markers including; CRP, AnxA1, IL-1β, TNF-α, Leptin and adiponectin and Gut hormones; PYY and GLP-1. The biomarkers will be measured using commercially available sandwich ELISA kits following the manufacturer's methodology at Coventry University.

Typical Sandwich ELISA methodology:

The capture antibody is coated onto a solid support; 96 well plate. The antigen is incubated with a primary antibody, followed by a wash with an appropriate buffer to remove any excess, unbound antigens. The primary antibody is incubated with an enzyme labelled secondary

antibody. The most widely used enzymes for detection are Horse radish peroxidise (HRP) and alkaline phosphatase (ALP). The enzymes are incubated with their appropriate substrates; P-Nitrophenyl-phosphate for ALP and hydrogen peroxide for HRP, and the chemical reaction result in change of colour. The amount of colour produced is quantified using a spectrophotometer plate reader at a target wavelength and is proportional to the amount of antibody/antigen present.

Oral Glucose Tolerance Test:

The OGTT is specific to this study, therefore, only a subset of participants will undergo this test. Participants who consent participating in an OGTT will be made aware that they need to abstain from ingesting food and fast from the previous night (fast >10 hours) will be required. 2.7mL of fasting blood sample will be collected by venepuncture in a grey top tube (containing sodium fluoride; an antiglycolytic agent) and stored on ice. It will be recorded as 0 minutes. Participants will be given 75g anhydrous glucose in the form of a drink which they will need to consume within 5 minutes. Second blood sample (2.7mL) will be collected by venepuncture at +120 minutes and stored on ice. If any glucose is lost by vomiting, the test will be discontinued. The samples will be sent the routine hospital lab for analysis of blood glucose, insulin and C-peptide levels. The OGTT will be carried out by a trained professional.

Measurement of insulin sensitivity

The homeostasis model assessment (HOMA) will be used to estimate steady state β-cell function (%β) and insulin sensitivity (%S) preoperatively and postoperatively.

The following equations are going to be used to calculate %S and %β:

$$HOMA\%\beta = \frac{20 \times Insulin}{Glucose - 3.5}\ \%$$ (Glucose in Molar Units mmol/L)

$$HOMA\%S = \frac{Glucose \times Insulin}{22.25}$$ (Glucose in Molar Units mmol/L)

Data collection

All data will be collected on a standard clinical records folder and then entered on to a secure database. All subjects will be allocated a study number and no identifiable information will be

transferred on to the data base. Access to the data will be restricted to the study team and will be password protected.

Statistical analysis

The statistical analysis will be carried out using one way Anova, Tukeys post hoc test and non-parametric spearman rank correlation. Inflammatory Biomarkers and Gut hormones will be analysed by ANOVA. The analysis will be performed using Graphpad and the level of significance will be set at $P< 0.05$.

Confidentiality

All the patients' data will be coded so that no personal identifiers are on the same database as the results from biochemical, gut hormones or inflammatory biomarker analysis. All databases will be password protected and kept on passworded computers in locked rooms. Hard copy of the original data will be kept in sealed enveloped in locked filing cabinets. Only the people directly involved in the project will have access to any of the results from the project.

Benefits of the project

The aim of this project is to investigate the relationship between the levels of AnxA1 and weight loss and the results of this pilot project will contribute to our understanding of the link between anti-inflammatory markers and obesity. Therefore, AnxA1 could be used as a biomarker and could aid in the development of effective therapeutic strategies to help overcome obesity associated inflammation.

The outcome of the pilot project can lead to investigating the area further using a bigger cohort and investigating a range of different markers. Furthermore, different populations of individuals can be recruited including those who have undergone surgery, have had effective weight loss, but have relapsed diabetes type 2.

Resources and costs

This project is funded by the Knowledge Exchange Unit at Coventry University through their SPIDER project. The main resources required for this project will be ELISA Kits to measure different biomarkers. Each kit is approximately £500 (96 tests).

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Patient Information Sheet

Plasma Annexin A1 levels and insulin sensitivity during weight loss through Bariatric Surgery

CU Ethics Reference Number: P50481

IRAS Ethics Reference Number: 220666

Sehar Sajid, Professor Derek Renshaw, Dr Srikanth Bellary, Helen Jenner

You are invited to take part in a study regarding obesity. Thank you for considering helping with our research. This form explains what you will be asked to do. If you have any questions

about this, please ask me. The project is being carried out as part of my PhD under the supervision of Dr S Bellary and Professor D Renshaw.

By signing this form, you agree to take part in the study. However, please note that you are free to stop taking part at any time. Blood samples and data collected with your consent prior to withdrawal will be used in this study. You will be given a copy of the consent form to keep for your records.

Information about the project/Purpose of the Project

My PhD project involves investigating the role of an anti-inflammatory protein; Annexin A1 in obesity associated inflammation. Obesity alters the level of inflammation and biomarkers in human blood. To further my project and our understanding of the relationship between inflammatory biomarkers and obesity, I am proposing a study in which I will be investigating the levels of Annexin A1 during weight loss through bariatric surgery and whether it increases following weight loss. Additionally, I will be correlating it with inflammatory biomarkers, gut hormones and insulin sensitivity.

Why have I been chosen?

We are looking for volunteers who will be undergoing bariatric surgery and I understand you are scheduled for the surgery soon therefore, your involvement and participation in this project will be highly valuable.

Do I have to take part?

If you don't want to take part, you don't have to give a reason, and it won't affect the care you receive. If you do want to take part now, but change your mind later, you can pull out of the study at any time. If you agree to take part in this study, you will be asked to sign the Consent Form on the last page of this document. This document is 5 pages long, including the Consent Form. Please make sure you have read and understood all the pages. You can discuss it with your family, friends or GP if you wish. You will be given a copy of both the Participant Information Sheet and the Consent Form to keep.

What do I have to do?

You will be asked to visit the clinic at Heartlands Hospital on 3 separate occasions; 1) before your surgery which will be around the time of your pre-operative assessment 2) 6 weeks after your surgery and 3) 3 months after your surgery. To minimise the inconvenience for you, the visits will be arranged in advance with you to coincide with your clinical visits.

During each of the visits which is expected to last between 1-1.5 hours, you will undergo basic clinical assessment in which your height, weight and blood pressure will be measured. A venous blood sample will be taken for biochemical analysis such as glucose, insulin and lipids by a trained member of staff. A separate venous blood sample will be collected for analysis of inflammatory markers and gut hormones to meet the aims of this project.

If you agree to take part and give your consent, you will be asked if you would like to participate in an additional test; the oral glucose tolerance test (OGGT). If you agree to take part, you will need to undergo this test on the 3 separate visits mentioned above. This test takes 2 hours to complete, so your visit is expected to last up to 3.5 hours. It involves coming into the clinic after an overnight fast, where you will have blood taken followed by a drink containing 75g glucose. The blood test will be repeated after 2 hours. The blood samples will be measured for glucose, insulin and c-peptide measurement You may experience some side effects which include nausea, stomach discomfort, diarrhoea and/or constipation as a result but you will be monitored throughout. This test is designed to test your body's ability to use sugar and can be used to diagnose prediabetes, diabetes and the other markers of insulin resistance. The participation in this test is optional and will not affect your decision to take part in the study.

Some individuals can feel dizzy or faint when they have needles inserted into veins. If you have previously fainted whilst giving blood, then please speak to me before you take part in the study. All participants will complete a standard laboratory health screening and risk assessment form prior to the sessions.

What are the benefits of taking part?

There are no extrinsic benefits for taking part in this study, however the information gathered will be important for future scientific studies which investigate role of Annexin A1 in adiposity.

Data protection and confidentiality

Your consent to participate in this study will be confidential. I assure you that complete confidentiality of data gathered in this investigation will be maintained. Your identity will not be made public without your prior consent. The samples will be transported to Coventry University in a secure boot of a car in an icebox and stored in locked -80°C Freezers. To ensure anonymity and confidentiality, the data will be coded and the original data will be stored in sealed envelopes in locked cupboards and passworded computers. Only myself and my supervisors will have access to the data collected and it will be destroyed within 5 years. At the expiry date, the samples will be incinerated and the sealed envelopes will be destroyed securely by shredding, by the Faculty Research Support Officer. The results of the project may be published in scientific journals and will be used in my Thesis which will be available through the Coventry University Library. A copy of the results can be made available upon request by contacting me once the project has been completed.

All information which is collected during the course of the research will be kept strictly confidential and comply with the principles of the Data Protection Act (1998). Please note that only the experimental team will have access to any data with your name on it. Any data stored electronically will use subject codes to retain anonymity. All data subsequently presented by these staff will be anonymous such that only these two staff will be able to determine that the data was gained from you. This study has been approved by the Coventry University – Faculty of Health and Life Science Ethics Committee and NHS ethics through IRAS. If you have any queries about this research, please ask;

Who should you talk to if you have questions

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Thank you,

Sehar Sajid

IRAS project ID	220666

Coventry University

Priory St,

Coventry,

CV1 5FB

Patient Identification Number:

CONSENT FORM

Title of Project: Plasma Annexin A1 levels and insulin sensitivity during weight loss through Bariatric Surgery

CU Ethics Reference Number: P50481

IRAS Ethics Reference Number: 220666

Name of Researchers: Dr S. Bellary

Professor D Renshaw

Sehar Sajid

Helen Jenner

Please initial all boxes

1. I confirm that I have read and understand the information sheet dated **01/08/2017** (version **2**) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that relevant sections of my medical notes and data collected during the study, may be looked at by individuals from Coventry University, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

4. I allow the researcher to inform my GP about my participation in the study ☐

5. I agree to take part in the Oral Glucose Tolerance Test ☐

6. I agree to withdrawal of blood samples ☐

IRAS project ID	220666

7. I agree to take part in the above study. ☐

Name of Participant	Date	Signature
Name of Person taking consent.	Date	Signature